

Living with nuclear radiation

The British reprocessing plant at Sellafield, long the undeserved whipping-boy of the anti-nuclear movement, is now the source of an unexpected consequence for children's health that will have international repercussions.

THE nuclear industry, already in the doldrums, will be set back on its heels again by the recognition that the relatively high incidence of childhood leukaemia near the British reprocessing plant at Sellafield may be the result of the occupational exposure of male workers to radiation (see pages 679 and 690). And the consequences will not be confined to Britain. Wherever people are occupationally exposed to radiation, there will (or should) now be a search for evidence of similar effects. That radiation can cause gross chromosomal abnormalities (recognizable, for example, in the circulating lymphocytes of those exposed) and, presumably, more cryptic genetic defects is, of course, well-known. The surprise is that the radiation exposure of fathers in advance of conceiving children should be a plausible explanation for the cluster of childhood leukaemia cases there and around some other British nuclear plants.

It is nevertheless important that the outcome of the new study should not be exaggerated. It dealt with 74 cases of leukaemia and non-Hodgkin's lymphoma among people younger than 25 over a period of 36 years (between 1950 and 1985) living within the territory of the West Cumbria Health Authority, which includes the Sellafield reprocessing plant. The numbers are small enough to be compatible with the assumption that leukaemia or lymphoma among the young is not intrinsically more likely at Sellafield than elsewhere in Britain, but may have been a chance event. So much was established by the investigation under Sir Douglas Black (now Lord Black) mounted in 1984 in the wake of a notorious television programme attributing the excess deaths to radioactive waste discharged from the plant.

In principle, there is no reason why the new developments should affect the conclusion that the occurrence of leukaemia near Sellafield is explained by chance. In practice, it will (see page 690). The consequences for the operators of nuclear plants, not only in Britain, will be considerable. Although it is not yet proven that radiation exposure of adult males sometimes leads to the birth of children with leukaemia, the reported association is bound to elevate the status of the connection from that of an untested hypothesis to that of a presumptive mechanism. Rightly, there will be pressure further to reduce occupational exposures, from workers and those who represent them. With hindsight, it will probably emerge that the British operators, British Nuclear Fuels Limited

(BNFL), have spent too much effort and money on the reduction of discharges and too little on reducing occupational exposure to radiation. Good sense would also dictate that public anxiety about waste disposal should be moderated.

An urgent need will also quickly be felt for a fuller understanding of the reported association. Does the likelihood of fathering a leukaemic child increase with the accumulation of radiation dose, or is the chance determined by exposure only in the few months immediately before conception? Questions such as these must be answered before individuals' exposure to radiation can be intelligently managed. And what explains the other leukaemic clusters recognised in Britain, many of them miles away from nuclear plants?

Britain, luckily, has an excellent system for the registration of deaths and their causes, and BNFL's records of occupational exposure are enviably detailed and complete. These circumstances provide an opportunity, perhaps unique, to arrive at answers. The Sellafield study, itself the fulfilment of one of the Black committee's recommendations, has required access to data that would ordinarily not have been available for proper ethical reasons. Much of what now needs to be done should ideally be less constrained. Especially because those concerned, BNFL in particular, appreciate the need for counselling the individuals involved, it is to be hoped that worthwhile investigation will not be needlessly impeded. □

Goodbye to junk?

The bankruptcy last week on Wall Street of Drexel, Burnham and Lambert should cast a long shadow.

DREXEL, Burnham and Lambert, Wall Street's upstart and most controversial investment bank, made its name, its own fortune and that of Mr Michael Millken, its Hollywood agent, by inventing the financial instruments called junk bonds. In old-fashioned America, bankers would lend money only against tangible collateral — real estate, perhaps, or saleable equipment and financial investments. Drexel's cleverness was to calculate that, for many companies, tangible assets are less important than expectations of future income. So a junk bond is a com-



pany's promise to pay out of future profits annual interest to those who buy its junk bonds. To allow for the risk that business might turn sour, interest rates must be higher than for bonds backed by tangible assets.

What is wrong with that? The simple answer is, or should be, "nothing". People who buy junk bonds are no worse off than people who buy the same company's stock over the counter of a stock exchange. If all goes well, the former will take the lion's share of profit and the latter what capital growth there is. There is much in Drexel's old boast that its unique invention made possible the reorganization of much of US industry and commerce in the interests of greater efficiency. So what went wrong?

Last year, Drexel was accused by the Securities and Exchange Commission not of inventing junk bonds (which cannot be a crime) but of manipulating the market in them. It pleaded guilty, paid a record fine of \$650 million and fatally exposed itself to lawsuits from all those who have lost money on its junk bonds. Millken has been indicted on similar charges, but plans to fight them. Among other things, it is charged that companies raising money by Drexel's successful issue of their junk bonds were induced to invest in later issues on behalf of other companies. One way and another, much of the junk has finished up on the books of the host of small savings banks in the United States, traditionally the custodians of individual savings, which have been attracted by high interest rates and by knowing that they would be insulated from much of the risk by the US government's guarantee of individuals' bank deposits. "Heads we win, tails you lose!" became the motto of the institutions still laughably known as "thrifts".

What, now, is to be done? Junk bonds are only part of the reason why many thrifts are in trouble, but the federal government's plan to rescue them is likely to cost US taxpayers \$150,000 million over the next decade. But that may not be the end of the story. Already there are rumblings that some company pension funds — also covered by federal guarantees — are over-invested in junk bonds. The reasons are obvious — high interest means the promise of high income, while the cost of companies' contributions to employees' pensions is reduced. The ultimate safeguard is the federal government's guarantee.

The fault is not that junk bonds exist — the opinion of many members of the Congress — but that the risks of owning them can so easily be minimized. What the Congress should now be doing is to hunt through the long list of guarantees against risk, or simple bad luck, it has provided to mortgagees, farmers and investors in small business as well as to pensioners and bank depositors, seeking ways of making the risks fall on the shoulders of those who take them. Otherwise, what the Drexel business has shown is that ingenious financiers will sooner or later find a way of turning each federal guarantee into a stream of risk-free investment funds. In the end, the result could bankrupt not just a single bank, but a large part of the United States. □

Soviet civil war?

The next few weeks will be more dangerous for the Soviet Union than any time since *perestroika* began.

THE two weeks ahead are days of suspended animation in the Soviet Union, perhaps inevitably. Mr Mikhail Gorbachev's triumph in persuading the Soviet Communist Party to relinquish its "leading role" was followed by its refusal, at least without further argument, to give him the power to run the Soviet Union as if he were its elected president. Nothing will happen now until after the elections at the end of next week, when the Russian Republic goes to the polls to elect local, regional and republic officials. The enforced hiatus will be a dangerous time, as will be the weeks that follow the elections.

The Party's faithful servants are certain to be further humiliated at the polls. Of that there seems no doubt. The difficulty, in present circumstances, is that their departure from office will leave a vacuum inadequately filled by the motley crew of nationalist and radical reformers seeking to succeed them.

The danger is that the Party, humiliated in polls it never sought, but which were wished on it by one of its number, and persuaded that the Soviet empire is almost beyond repair (which is the case), will take the opportunity to reassert the power it has enjoyed for the past 70 years. The trouble is that there are too many people in haertland Russia, not to mention republics in the south and north, who have liked their taste of freedom. People have muttered about the danger of civil war in earlier *perestroika* years, but not openly as now.

In a telegram to Gorbachev, a group of members of the People's Chamber of Deputies who are drawn together by their background in science (many of them are members of the Soviet academy) have now uttered a warning of the dangers as they see it. They are right to be alarmed. Well-disciplined because well-repressed, Russia has become a lawless place. People break into each other's houses and mug each other in the streets in ways that would have been unthinkable when the security services were full of bounce (and well supplied with informers). Anti-semitism, always latent, has become outspoken. The chilling concluding sentence in the telegram to Gorbachev is a reminder that in a civil war, there would be no way of telling how contending forces would use the threat of damage to nuclear reactors as a means of keeping the local people hostage. For that matter, nobody seems quite sure who has charge of the Soviet Union nuclear stockpiles.

The chances are, and the hope must be, that none of this will happen. But the danger is too vivid in too many hard heads to be overlooked elsewhere. There is not much that can be done at this late stage except to discover where one's friends are, and to offer them help if they should need it. It could come about that there will be a flood of takers. □

Leukaemia cases linked to fathers' radiation dose

- Environmental effects ruled out
- Government inquiry to begin

London

AN excess of childhood leukaemia cases in the village of Seascale, near British Nuclear Fuels' (BNFL's) Sellafield nuclear waste reprocessing plant, is linked in a report published last week to high radiation doses received by workers at the plant before their children's conception.

The association now reported is the first of its kind to be recorded in humans, although there have previously been reports that male and female experimental animals irradiated before mating produce damaged offspring. The occurrence of childhood leukaemia around Sellafield has been a matter of public concern since 1984, when the region was recognized as one of several in which there have been 'clusters' of childhood leukaemia cases.

The UK Department of Health, responding promptly to the findings published last week, has referred the study for "urgent consideration" by the Committee on Medical Aspects of Radiation in the Environment (COMARE), an independent scientific group which advises the government.

A research team led by Professor Martin Gardner, from the University of Southampton, looked at 52 cases of leukaemia and 22 of non-Hodgkin's lymphoma in the Sellafield area, diagnosed between 1950 and 1985, for statistical links to various factors thought to increase the risk of leukaemia.

By comparing the 74 cases with 1,001 healthy people taken from the same birth registers, Gardner and his colleagues ruled out environmental discharge of radiation from Sellafield as the culprit. Although there was some evidence for a connection between the children's leukaemia and their mothers' exposure to X-rays or viral infections during pregnancy, only their fathers' exposure to radiation while working at Sellafield can explain the tenfold excess in leukaemia cases at Seascale, says Gardner.

Gardner's results, published in the *British Medical Journal**, show that children were seven to eight times more likely to develop leukaemia if their fathers had received a total radiation dose of 100 milli-Sieverts (mSv), or more than 10 mSv in the six months before conception. Radiation doses were found from film badges worn by all BNFL radiation workers.

This is the first time that childhood leukaemia has been linked to fathers' occupational radiation exposure. A Japanese study (see *Nature* 296, 575; 1982) showing that male mice exposed to radiation father offspring are more likely to develop cancer has not previously been thought to have much bearing on human leukaemia, because it is contradicted by data gathered among Japanese atomic bomb survivors.

Those data show no excess of leukaemia cases among the children of the atomic bomb survivors. Although survivors of Hiroshima and Nagasaki suffered much greater radiation exposure than anyone at Sellafield, Gardner now suggests that the difference between the two groups may be explained by differences in the physiological effects of a single large exposure and the accumulation of lower-level radiation doses.

Nevertheless, some radiation scientists believe the Hiroshima and Nagasaki results weigh against Gardner's interpretation. Richard Doll, from the Imperial Cancer Research Fund's laboratory in Oxford, doubts whether radiation is the true explanation for the high leukaemia incidence at Sellafield, and suggests that occupational exposure to chemicals, happening to correlate with radiation dosage, may be involved. But Doll is pleased that Gardner's results focus attention on occupational exposure to radiation, rather than environmental discharges. The paper "takes the heat out of concern over radioactive waste in the environment", he says.

COMARE will consider the Sellafield results along with those from similar studies, now under way in the Dounreay area of Scotland (the site of BNFL's other reprocessing plant) and around the Ministry of Defence atomic weapons research establishments at Aldermaston and Burghfield. These will report at the end of 1990, or early next year.

Gardner's team is also planning further studies at Sellafield, relating fathers' occupational radiation exposure to leukaemia and other cancers for a much larger cohort of children born in the area. Unlike the present study, this will include children who moved away from Sellafield. Gardner is also looking at leukaemia rates in children born before 1952, when the Sellafield plant opened.

The new findings are a blow to BNFL, which already faces legal action from several parents of children with leukaemia from the Sellafield area. After discussing

ASTRONOMY

"Spinning pulsar" claims retracted

New Orleans

ASTRONOMERS who last year reported a pulsar that was apparently rotating at an unheard of rate of nearly 2,000 times a second have discovered an experimental error in their data that could negate the results. Speaking at the annual meeting of the American Association for the Advancement of Science this week, John Middle-ditch, an astronomer at the Los Alamos National Laboratory, said that interference from a video camera may have caused the spurious data. The pulsar's perceived rotational frequency of 1968.63 Hz corresponds to a characteristic video frequency.

Signals indicating the apparent pulsar had been spotted only once in the debris left after supernova 1987A (see *Nature* 338, 234-236; 1989), but subsequent statistical analysis of the data seemed to confirm the event. Such a quickly spinning pulsar would defy conventional theory and has sparked a number of new proposals in the hope of explaining it.

G. Christopher Anderson

the new results with Gardner, the company is to set up a joint investigation at Sellafield with the Health and Safety Executive's Nuclear Installations Inspectorate, which is expected to look at 'internal' radiation doses — radiation actually taken into the body, rather than external measures taken from badges attached to employees' work clothes. The internal exposure can be calculated retrospectively, using the known half-lives of radioactive isotopes. Occupational exposure to chemicals may also be studied.

In the meantime, BNFL says its medical officers will counsel any of the Sellafield workforce who are worried by Gardner's results. They also stress that they have taken measures to reduce their workers' exposure to radiation. The present UK legal limit for occupational exposure is 50 mSv per year, but the National Radiological Protection Board recommended in 1987 that doses should be kept below an average of 15 mSv per year wherever possible.

In 1988, 402 of the Sellafield workforce received more than 15 mSv, fewer than half the number in 1987. But Gardner's results have already led to calls for BNFL to take further action to reduce radiation exposure. Unions representing the Sellafield workers are to meet with Gardner and BNFL management during the next week. Bill Brett, general secretary of the Institution of Professionals, Managers and Specialists, which represents the majority of BNFL's radiation workers, says "there must be prompt examination of current radiation dose limits and further action to protect all employees".

Peter Aldhous

*Results of case-control study of leukaemia and lymphoma among young people near Sellafield nuclear plant in West Cumbria. Gardner, M.J., Snee, M.P., Hall, A.J., Powell, C.A., Downes, S. & Terrell, J.D. *Brit. med. J.* 300, 423-429 (1990).

NIH seek advice on Gallo

Washington

THE US National Institutes of Health (NIH) Office of Scientific Integrity last week made public that it was asking the National Academy of Sciences for advice in the preliminary stage of a 'fact-finding' inquiry into the conduct of National Cancer Institute researcher Robert Gallo.

Although details of the inquiry have not been revealed, Susanne Hadley, director of the Office of Scientific Integrity, says that the fact-finding process began last autumn. It was at this time that the *Chicago Tribune* newspaper published a 16-page supplement written by journalist John Crewdson on the famous dispute over the discovery of the AIDS virus. That dispute appeared to have been settled in 1987 when then President Ronald Reagan and French Prime Minister Jacques Chirac agreed to name Robert Gallo of the National Cancer Institute and Luc Montagnier of the Pasteur Institute as co-discoverers of the virus. But in his *Chicago Tribune* article, Crewdson alleges that the contribution of Gallo's laboratory could have involved an "an accident or a theft".

Hadley stresses that a formal NIH investigation of Gallo is not under way. The case, she says, is "in the inquiry stage right now . . . we are fact finding to see if an investigation is warranted." She says that NIH have asked the National Academy of Sciences to recommend a review panel but this does not mean that a formal investigation will necessarily follow. "We can assemble a panel for a number of other reasons", she says. NIH guidelines normally allow a 60-day period between the beginning of a fact-finding inquiry and a decision whether or not to proceed with a formal investigation, although Hadley says the deadline may be extended in some circumstances.

The Office of Scientific Integrity normally will neither confirm nor deny that an inquiry or investigation is under way. But Hadley says that an exception was made in this case because of its significance "for NIH and biomedical research".

The publication of the *Chicago Tribune* supplement did not attract much attention in the scientific press or in US newspapers last autumn as many of its allegations were considered to be already well known. But the supplement did stimulate Congressman John Dingell, chairman of the oversight and investigations subcommittee that last year conducted several hearings into the case of Nobel-laureate David Baltimore, to write to William Raub, acting director of NIH (see *Nature* 343, 3; 4 January 1990).

Dingell asked whether NIH were going to look to look into the *Chicago Tribune* allegations and was not satisfied with

Raub's reply that a panel from Gallo's own institute, the National Cancer Institute, would be set up. A further exchange of letters followed in which Dingell made more specific demands.

It is not known if these letters pushed NIH's Office of Scientific Integrity into asking the National Academy for Sciences for advice, but Hadley points out that the fact-finding process had begun before Dingell first wrote to Raub.

Gallo has been hit hard by renewed interest in the allegations, according to an account of a telephone interview with National Cancer Institute director Samuel Broder, published in the 18 February issue of *Out Week* magazine.

In the interview, the substance of parts of which has been confirmed by the institute, Broder says that "Bob [Gallo] is being tied up in knots. I can't talk science

with him. His mind is distracted and he can't focus on it".

Copies of the relevant page of *Out Week*, a small-circulation magazine providing strong support for homosexual rights, were sent by fax to several science journals and other media last week. It is not known who sent the fax messages or why, although whoever did so apparently took the precaution of deactivating the message line that identifies the sender's fax machine number. The copies were not sent by the magazine itself.

The *Out Week* article goes on to imply that Gallo is now under fire at the National Cancer Institute for lack of productivity.

But the author, Larry Kramer, who is frequently critical of the AIDS 'establishment', goes on to say that "For all his craziness . . . Gallo still knows more about AIDS than almost anyone around".

**G. Christopher Anderson
& Alun Anderson**

RECOMBINANT DNA REGULATION

India opts for self-control

New Delhi

SAFETY guidelines for recombinant DNA research just released by the Department of Biotechnology seek to regulate genetic engineering research in India more by self-control than by legal measures. Companies in countries such as West Germany where laws are strict may even find India's relaxed approach sufficiently attractive to shift some of their genetic engineering research to India.

The guidelines are the work of an 18-member recombinant DNA advisory committee led by S. Varadarajan, an industrial chemist and former chief of the Council of Scientific and Industrial Research. Interestingly, issues relating to genetic engineering of human embryos, use of embryos and fetuses in research and human germ-line gene therapy are excluded from the scope of the guidelines.

Under the new rules, recombinant DNA work is classified into three categories depending on the perceived risk, and taking into account local factors such as immunity to diseases, laboratory environment and tropical conditions. Approval by the competent authority is needed only for such experiments as cloning of genes for toxins and for vaccine production, gene therapy and field release of altered organisms. Most recombinant DNA experiments need only notification and not approval.

S. Ramachandran, secretary of the Department of Biotechnology, says the guidelines were meant to make researchers aware of the need for good laboratory practice. Stringent laws, he feels, will stifle work in this important area where India is just beginning to make progress.

The guidelines are to be implemented by a three-tier mechanism, but the onus of ensuring safety rests mostly with an in-house biosafety committee within the research institute itself.

Whether or not the institutions abide by the guidelines will be monitored by a Review Committee for Genetic Manipulation on the basis of surprise visits and bi-annual reports from the biosafety committees. Any violation, deliberate or due to negligence, will lead to cancellation of research grants — a threat of no consequence to private companies, Indian or foreign, which do not depend on government funds.

Legal action will be taken only in case of violations in regard to field release of engineered organisms or products. Such releases must have the approval of a genetic engineering approval committee to be set up under the Department of Environment. It will be a statutory body with "judicial powers to inspect, investigate and take punitive action" under the Environmental Protection Act, the same act that deals with air and water polluters. The act has not been successful in controlling industrial pollution because it contains many loopholes and imposes few sanctions.

Whether self-control is good enough to regulate recombinant DNA technology remains to be seen. According to Ramachandran, some 26 institutions have set up biosafety committees and others have been asked to fall in line. He admits the new rules are not fool-proof but is confident that deliberate violations of the guidelines cannot remain undetected for long.

K. S. Jayaraman

Mixed reactions to merger

Washington

THREE weeks after Genentech Inc. announced its 'marriage' to the Swiss-based company, Roche Holdings Ltd, biotechnology executives and stock analysts have mixed opinions, but no one is predicting a wave of merger mania among biotechnology companies. The Genentech deal gives Roche a 60 per cent stake in the South San Francisco-based company in return for a \$2,100 million investment (see *Nature* 343, 495; 8 February 1990), providing Genentech with the capital to pursue an ambitious drug development programme free from Wall Street's pressure on quarterly earnings.

Many people expected Genentech, the first biotechnology company to go public (in October 1980), to become the next major independent drug-producing company in the United States, but the Roche deal has emphasized the capital-intensive nature of the industry. It can take 7–10 years and an investment of \$125–\$150 million to bring a drug to market. Lowell Sears, chief financial officer at Amgen, points out that there is a "lack of appetite for investment [by US financial markets] in the future of biotechnology" and that "the management at Genentech had grown weary of the scepticism and lack of commitment from financial markets". "Equity investors still consider biotechnology to be a risky business", says Pamela J. Bridgen, executive director of the Association of Biotechnology Companies. Consequently, Genentech stock was probably undervalued.

The Genentech-Roche merger provides, says Katharine Russell of Cetus Corporation, an "outside-the-industry validation" of biotechnology. Clearly, Roche has put a value on the two Genentech products already on the market — the blood clot-dissolving drug Activase (tissue plasminogen activator, TPA) and Protropin (human growth hormone), and on its valuable longer-term pipeline of products. John Girton, a San Francisco stock analyst, says the share prices for Amgen, Inc., Chiron Corporation and Cetus Corporation rallied after the 2 February announcement. The news, he says, has introduced a "take-over premium" into other companies.

Indeed, four days after the Genentech announcement, Cetus went back to the equity markets for an injection of capital. It made a secondary issue of 2.6 million common shares — money that Cetus says is earmarked for financing clinical testing and improving its sales and marketing infrastructure. But Denise Gilbert, a San Francisco analyst, warned that this optimism may be premature. This is a "one-of-a-kind deal" because "Genentech has assets that no other company has".

Genentech has fewer strategic alliances with other companies and has retained the marketing rights to its own products. Gilbert believes that the deal will make Genentech more formidable and, consequently, the rest of the industry must look over its shoulder to see what Genentech will do. One possibility is that Genentech may try to obtain the marketing rights to other company's products.

But Girton disagrees, saying that although Genentech was particularly attractive, it was not unique. He cites Amgen, Cetus, Immunex, Genetics Institute and Biogen as also having valuable long-term product pipelines.

Richard D. Godown, president of the Industrial Biotechnology Association, observes that in 15 years of biotechnology, the Genentech-Roche deal is only the third major acquisition of a biotechnology company. A wave of buyouts was predicted in 1986 when Eli Lilly & Company bought Hybritech and the Bristol-Myers Company acquired Genetic Systems Corporation, but did not materialize. Godown points out that Genentech was not forced into the merger and that discussions were initiated by the company. It was not because of "pressing economic necessity, but as a matter of corporate planning", says Godown.

This sentiment is echoed by Girton, who says that Genentech was "funding about \$145 million of R&D a year and was profitable to the tune of \$40 million a year". He believes that the venture capitalists "cashed out" and put a "ceiling limit" on the share price. No one is going to pay more than \$38 per share this year, knowing that Roche can exercise its right to buy outstanding shares at that price.

The latest deal gives Roche access to the two most promising areas of biotechnology. It already owns a 4.5 per cent stake in Cetus, and its wholly owned US subsidiary Hoffman-La Roche of Nutley, New Jersey, is licensed to develop diagnostic products using Cetus's PCR (polymerase chain reaction) technology. Whether Genentech can maintain an arms-length relationship with Roche remains to be seen. Girton sees Genentech becoming a division of Hoffman-La Roche, and Godown says it is "reasonable to assume the inevitable coming together of Hoffman-La Roche and Genentech".

There has been little political comment over the fact that a controlling interest in the leading US biotechnology company is in foreign hands. Representatives Rick Boucher (Democrat, Virginia) and Carlos Moorhead (Republican, California), recognizing that too many home-grown industries are being swallowed up by foreign competitors, have introduced a bill to stop unfair foreign competition in

Biologist Phillip A. Sharp chosen

Boston

PHILLIP A. Sharp, a well-known cancer biologist, has been selected as the new president of the Massachusetts Institute of Technology (MIT). Although the decision will not be confirmed officially until a vote by the MIT Corporation on 2 March, the choice has already been approved by MIT's search committee and faculty advisory committees.

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Professor Phillip A. Sharp — new MIT president.

Sharp, 46, is currently director of MIT's Center for Cancer Research and will be the first biologist ever to head the university, a sign of the increasing prominence of biological research at the institute. He is known for the discovery in 1977 of surplus DNA and the splicing of RNA, and was a 1988 Lasker award-winner. He is also a co-founder and board member of the biotechnology company Biogen, an affiliation that is said to have earned him millions of dollars' worth of stock.

Sharp's selection caused some surprise at the university: he is a scientist, not an engineer, and works in the life sciences rather than the physical sciences. He is a well-known researcher but has had little involvement in the university's administration. Some members of the selection committee said that they hoped these attributes would allow Sharp a "fresh opportunity" to examine the school and to exert an influential voice on behalf of the scientific community. Sharp has declined to speak publicly about his new post until he is officially confirmed.

Two prominent early candidates for the presidency of MIT dropped out of the competition. David Baltimore, one of Sharp's closest colleagues, recently accepted the presidency of Rockefeller University in New York, and MIT Provost John Deutch withdrew his name earlier this month, after controversy about his corporate and military ties (see *Nature* 343, 585; 15 February 1990). Seth Shulman

biotechnology. The bill seeks to eliminate certain unfair advantages that US patent law now confers on foreign competitors. But asked about the Genentech-Roche deal, Boucher said only that he regretted both that it had happened and that it had become necessary.

Diane Gershon

Donna Coveney

Researchers begin life again

Bucharest

AFTER 25 years of the Ceausescu dictatorship, researchers in Romania again have hopes of progress. The provisional government plans to improve the lot of basic research and the first concrete steps in that direction are now being taken.

The National Salvation Front, which took power from dictator Nicolae Ceausescu in the December revolution and set up a provisional government, has endeared itself to researchers by restoring cuts in their salaries made by the Ceausescu regime.

In industry, as well, workers had to 'loan' the government up to 40 per cent of their pay for months at a time in order to build new factories. These cuts, too, have been restored.

There is some scepticism about the promises of the Front. Its nickname among dissenters is the *Nomenklatura* Salvation Front, *nomenklatura* being the leading Communists from the old regime. And even the Deputy Minister for Environment, M. G. Negulescu, admits that with the Front issuing "ten decrees a day", it is hard to know what to believe. But perhaps because they have no choice, researchers trust the Front when it says it has the best interests of science at heart.

Dan Balteanu, the new democratically elected director of the Institute for Geography affiliated with the Education Ministry, said the Front sees supporting science "from below" in a decentralized manner as a "noble thing". Balteanu was especially encouraged when the Front almost instantly disbanded the hated National Council for Scientific Research (NCSR), which used to run Romanian science under the watchful eye of Elena Ceausescu, the dictator's wife.

Two weeks ago, the Front issued a decree including a new set of by-laws for the Romanian Academy. Most important among these, say researchers, were the articles granting autonomy to the academy and stating that its main purpose is supporting basic research.

This is a complete turnaround from the policy of the Ceausescu regime, under which the NCSR severed the academy from its 51 research institutes (including the Institute for Geography). The institutes had to turn more and more to applied research in order to function as their support was cut. According to the Front, many institutes will be restored to the academy later this year and their support will be guaranteed.

The Front set up a 'Salvation Committee' for the academy just ten days after the revolution. Led by Professor Milcu, an 85-year-old endocrinologist, the committee prepared a secret ballot among the academy's remaining members, whose

average age is over 75, to elect a president. Their unanimous choice, Mihai Draganescu is an information scientist whose institute was abolished in the 1970s. He is also a vice-prime minister in the provisional government.

The changes in the academy do not make everyone happy. A few of its previous administrators have resigned to make way for the new leadership but in the institutes themselves there is a "fantastic power struggle all over", reported one researcher at the Institute for Biology. People instrumental in the old regime are "fighting tooth and nail" to hang onto their positions, said Nicolae Botnariuc, the new head of the biology section in the academy. Not only are they concerned about losing their salary or their title; many of them are so incompetent that they "would not be able to cope with life outside of their institutes". The Ceausescu regime tried to kill intellectual talent. "They limited us in any way they could", said Serban Dragomirescu of the Institute of Geography. Personnel, training, transportation, documentation, foreign travel and even simple office equipment were subject to exasperating restrictions; the typewriters in the institute could be used only with permission from the NCSR. The latest piece of equipment was bought for the institute in 1975.

Even Romanian-produced photographic film was made inaccessible to the geographers a few years ago because of an ostensible shortage of silver. Film and other supplies sent by foreign colleagues could not be received without going through humiliating formalities, said Dragomirescu, so it often never reached the institute.

Collaborations were effectively stopped by the regime, which forbade researchers from travelling or receiving visitors or even answering letters from abroad. Needless to say, researchers are desperate to re-establish the contacts they once had or to establish new ones.

"We could not even exploit the talent we had in the country", lamented Dragomirescu, because the regime allowed the institute to recruit new employees only from Bucharest.

The geographers learned to be grateful for small victories. They displayed with pride a copy of the Romanian National Atlas, which was published by the institute in 1979. They are not only proud of the oversize, colourful atlas — they considered it a moral victory that 10,000 copies were produced and not one has a photograph of Ceausescu in it.

A few researchers were sceptical of the new academy plan because of the implicit centralized structure that it imposes on basic research. S. A. Hulea of the Institute

Scholarships offered

New Delhi

INDIA will provide scholarships to 500 undergraduate and postgraduate Sri Lankan students whose studies have been interrupted by the closure of educational institutions amid continuing ethnic violence. The students, including Sri Lankan Tamils, will be placed in various universities and colleges in India during the 1990–91 academic year.

Professor Cyril Ponnemperuma, scientific adviser to the Sri Lankan president, who was recently in India to attend a science congress, said that an estimated 18,000 students have been affected by the closure of most of the nine universities in Sri Lanka. The government approached several countries to ask if they would accept at least 1,000 students but, so far the only offer is from India which has agreed to take 500 of them. Ponnemperuma said the offer by India will strengthen Indo-Sri Lankan ties and that "what the countries failed to achieve at the political level, might be achieved at the educational level".

The project will cost India about \$3 million a year and will be administered by the University Grants Commission and the Indian Council of Cultural Relations. According to the chairman of the grants commission, Professor Yash Pal, the External Affairs Ministry will mobilize the needed financial resources.

K. S. Jayaraman

of Biology said that Romania needs a "new model" for supervising research. "But we don't know what it is", he said. "We are still waiting for instructions from someone. After 45 years of following the orders of authority, it is hard to change ourselves within one month." People are aware that Romania has more than a little catching up to do. Botnariuc of the academy said that "Romania's main problem is not the economy, which can be rebuilt with some effort. The biggest damage [of the dictatorship] was to people's psyches and their intellects. All the important things in their lives were distorted — morals, ethics, their mentality. These are the hardest to change because they go so deep."

The period of uncertainty will probably last well beyond the elections scheduled for May. Many people see the need for more shaking out of Ceausescu sympathizers before the scientific institutes, or the society as a whole, can go on in peace. As Hulea put it, "people who were quiet here for a long time are suddenly revolutionaries. It's very strange." A story going around Bucharest has it that Diogenes came to Romania this month looking for someone who did not participate in the revolution. He has yet to find one.

Steven Dickman

CONFERENCES

Meetings in north and south

New Orleans, Louisiana & Hobart, Tasmania

BOTH the American Association for the Advancement of Science (AAAS) and the Australian and New Zealand Association for the Advancement of Science (ANZAS) opened their annual congresses last week. Both chose to give most weight to the predictions and possible effects of global changes in climate; ANZAS with 'Global change and the South-west Pacific' as its theme, and AAAS with global change as one of the three broad areas into which the conference was divided.

Both conferences were also marked by disappointing attendance figures. ANZAS in Hobart attracted only 700 people and AAAS in New Orleans around 6,000, including the 500 press, 1,000 speakers and many high-school students who attended special events. Part of the problem is location; neither Hobart nor New Orleans has a large local scientific community. At San Francisco last year, AAAS drew 10,000 people.

Scientists in both nations rehearsed gloomy scenarios to accompany possible global temperature rises but only the Australians managed to find a little cheer amidst the predicted mid-twenty-first-century disappearance of the Marshall Islands and Tuvalu beneath the rising seas.

Barrie Pittock, from the Commonwealth Scientific and Industrial Research Organization (CSIRO) Division of Atmospheric Research in Melbourne, estimated that US grain exports, which account for 50 per cent of the international market, would be hit by severe drought. But global warming would boost grain production in all Australian states except Western Australia, providing Australia with a chance to increase exports.

Both conferences had their share of eccentricities and disagreements. As talk titles go, ANZAS wins the prize for "Premises for the feasibility of motors using principles of psychokinesis". In New Orleans, the debate over the role of Albert Einstein's wife, Mileva Maric, in the formulation of the theory of relativity looks set to run and run.

At AAAS, a session on cold fusion could still pack a room and leave a small crowd listening at the door, but it was no longer scientific miracles that people were there to hear. The cold fusion affair is already a case study and the speakers were two sociologists, two historians and one physical scientist. The National Science Foundation announced that it is assembling a cold fusion database at Cornell University to record the event and its backlash. Already more than four cubic feet of electronic mail messages, laboratory and lecture notes, press clippings and

records of press conferences have been assembled.

Other than the many aspects of global change, half a dozen topics among 200 odd sessions spread over five days were considered weighty enough for a full day of talks at AAAS. Human sexuality and sexual behaviour was one of them, and the results of new US national survey by Tom Smith of the University of Chicago drew a packed house. To everyone's surprise, Smith found that US adults are far more puritan than previous surveys and anecdotal evidence would suggest.

According to the survey, 22 per cent of Americans were abstinent last year, and only 1.5 per cent had an extramarital affair. "Chaste" adults who had never had sex with anyone other than their marriage partner account for nearly half the population.

Less than 1 per cent of adults are exclusively homosexual, and only 1.5 per cent reported any homosexual contact in the past year. The homosexuality figure is nearly an order of magnitude lower than previous estimates. Although the study sample size was just 1,400, the data suggest that only 6.8 per cent of the population is at a relatively high risk for AIDS infection, Smith says.

POLAR RESEARCH

French plan new initiative

Paris

AT a cabinet meeting last week, research minister Hubert Curien and Louis Le Penec, the minister for overseas departments, offered new proposals to reorganize French polar research. An inter-ministerial communiqué argues that the importance of the polar regions for the study of climatic change, astronomy and, in the Antarctic, the ozone layer, requires France to increase its activities.

In 1989, France spent FF110 million (\$19.3 million) on polar research, about half of what Britain or West Germany spent. At present, French polar research is concentrated in the Antarctic, with a single base under the administration of the TAAF (the French Austral and Antarctic Territories), a government body not geared to research. Under the new plans, an 'Institute for Polar Research - Paul Emile Victor Expeditions' will be set up, with interests in both polar regions.

Initially, the institute will be financed out of the existing budget for polar expeditions but in the medium term research money will be diverted from the TAAF, together with an extra FF10 million per year for Arctic research from the research ministry. The long-term goal is to attract private sponsorship to transform the

Another full day went to the use of animals in biomedical research, with AAAS taking a tougher stand as an advocate for animal research. Frederick Goodwin, head of the Alcohol, Drug Abuse and Mental Health Administration, says that there has been a serious decline in the number of papers published in which primates are used for drug addiction research, the area that has been one of the biggest targets of the animal rights movements.

The most powerful message came from Steve Carroll of Incurably Ill for Animal Research. His own life was saved after a plane crash as a result of medical advances made through animal research and he now runs a group that tries to publicize the benefits of medical research.

But among researchers, discussion of a moral position appears to be only just beginning. Animal rights campaigners are much more sophisticated and Goodwin estimates that they have \$50 million a year to spend on attacking the use of animals in biomedical research alone. Most of the money is raised in direct mail campaigns. Goodwin promised to get tough with institutes that did not protect their researchers, saying that he may have to examine grants to those that bow to pressure from animal rights campaigns.

**Tania Ewing, G. Christopher Anderson
& Alun Anderson**

institute into a charitable foundation.

Two new interministerial committees will be established. One will make strategic decisions and will decide the main budgetary priorities, while the second will be a polar environment committee for ensuring "the compatibility of research activities with environmental conservation, in keeping with the position adopted by France during the fifteenth consultative conference on the Antarctic Treaty in Paris last October".

Although one goal of the new proposal is to coordinate research in both polar regions under a single administrative banner, most interest will still reside in the Antarctic. The present summer base at Dumont d'Urville is, says the report, "running out of breath". A second base, 1,000 km inland, is planned for 'Dome C', a 3,200-metre-high plateau near the South Pole and directly under the hole in the ozone layer. Dome C is in the Australian Territory and the French hope to use the abandoned infrastructure of a previous US camp there.

The base will be the third inland station, along with the US South Pole base and the Soviet Vostok base in the Antarctic highlands, and will allow 15 scientists to winter on the continent.

Peter Coles

"More research needed"

Washington

THE Bush administration, under fire from environmentalists for its reluctance to embrace global warming counter-measures, believes that two more years of research is needed before the science of climate modelling is strong enough to dictate environmental policy, said Energy Secretary James Watkins in an interview last week.

By 1992, "our national laboratories are going to have enough answers for us to get specific" about climate predictions, Watkins said. The administration reckons it will be spending half the worldwide total on climate change research in 1991; Department of Energy (DOE) research alone accounts for \$66 million of the request.

Although several European countries have already begun programmes that would restrict the emission of carbon dioxide and other greenhouse gases to current levels, Watkins said recent scientific advances still cannot "economically justify taking drastic steps" to control emission in the United States. But he believes that the moderate measures already in place can have a significant impact on greenhouse gas concentrations. "We're going after all the knowns, such as acid rain, the ozone layer, and deforestation, while we wait for the science clarification" on global warming, he said: efforts such as the phasing out of chlorofluorocarbons, along with new energy-efficiency initiatives, can cut greenhouse gas emission by about a quarter in the next decade.

Speaking a week after President Bush opened a United Nations meeting on climate change by calling cautiously for more research, without accepting predic-

economy for world survival?"

"Although he said he is "not against the thrust" of minimizing greenhouse gas generation, he objected to "premature" congressional bills by Senator Tim Wirth (Democrat, Colorado) and Representative Claudine Schneider (Republican, Rhode Island) that would require a 30 per cent reduction in carbon dioxide emissions by the end of the century. "To solve something out of the hip pocket like that in an election year may be attractive, but I think it is not the responsible approach", Watkins said.

Watkins believes that increased use of hydroelectric power and "biomass" fuels such as ethanol could fulfil three quarters of the new energy needs in the next decade. "Perhaps with those two things and conservation, we could get very close to hold-

ing the line on current emissions" without imposing additional controls on industry, he said. Conservative administration officials such as White House chief of staff John Sununu have fought bitterly against new industrial emissions regulations.

In private debate within the administration, Watkins is said to side occasionally with William Reilly, the director of the Environmental Protection Agency, who has seemed to fret against the cautious approach of the Bush administration. But in public Watkins remains mindful of the party line. He refuses to use the term "global warming", for example, preferring to talk about "climate change".

"Obviously there are some environmentalists who are never going to be satisfied [with administration policies]. But we're trying to cut a wide swath down the middle of this thing and I think we're doing a pretty good job of it", he said.

G. Christopher Anderson

HUMAN FRONTIERS

Applications under review

Tokyo

THIS week, international committees of scientists in Strasbourg will begin reviewing a flood of hundreds of applications from scientists around the world to Japan's Human Frontier Science Program which was established as a foundation in Strasbourg last November.

Frontiers, which is funded almost entirely by the Japanese government, provides grants for international teams of scientists, fellowships and support for workshops for research on the brain and molecular recognition. The programme is open to applicants from any country in the world but the principal researcher for grants must belong to one of the seven summit nations (United States, Canada, United Kingdom, West Germany, France, Italy and Japan) or a non-summit European Community (EC) nation. And fellowship applicants from non-summit nations must carry out their research in a summit nation (summit nation fellowship applicants can go to any country they wish other than their own).

Frontiers is proving very popular. Eight hundred and twenty-five scientists from 33 countries have made 236 applications for the approximately 20 \$500,000 three-year grants available this fiscal year. And 210 young scientists have applied for the 100 long-term fellowships (but only ten have applied for the 50 short-term fellowships). There are also 35 applications for support for workshops, ten of which will be accepted.

Applications will be screened by four committees each comprising 16 eminent scientists (two from each summit nation and two from the EC). Two committees (one for brain research, and one for

molecular research) will screen the grant applications after a preliminary peer review by mail, and the other two committees will review the fellowship and workshop applications. The awards are expected to be announced in the middle of March after approval by the programme's council of scientists.

Ironically, it is countries contributing no financial support to Frontiers that stand to gain the most if the awards reflect the distribution of applications. The biggest number of grant applications (66) comes from principal scientists based in the United States as does the biggest number of fellowship applications (66). The United Kingdom also has a large number of applicants for grants (47) and fellowships (26). Japan, with 56 grant and 38 fellowship applications, fares comparatively badly considering that Japan is financing most of the programme.

Another feature apparent from the applications is that the United States is by far the most popular destination for fellowship applicants. More than half the applicants (107) want to go to there. On the other hand, only ten young scientists want to go to Japan and nearly four times as many want to leave (38).

Toichi Sakata, director of the Frontiers office at the Science and Technology Agency (STA), hopes that more countries will agree to contribute funds to the programme now that the framework for Frontiers is complete and nations can see how it works. He says the lack of fellowship applicants to Japan is disappointing and STA will take measures to publicize the strengths of Japan's research system to try to attract more foreign scientists.

David Swinbanks

Read my lips!...um... well....

that is...
er....



tions for global warming (see *Nature* 343, 500; 8 February 1990), Watkins acknowledged that DOE's own experts do indeed forecast a global temperature rise from present predictions of the increase in carbon dioxide. "I believe that there is a greenhouse gas increase, and infrared absorption [by those gases]. The [DOE] labs clearly say that the greenhouse effect is generated under those circumstances," he said. But, he added, do "we have to destroy the industrial base and our

Fine images from NTT

Garching, West Germany

FIRST results from the New Technology Telescope (NTT) of the European Southern Observatory (ESO) in La Silla, Chile, bode well for its new 'active optics' technology and for the success of ESO's next big project, the Very Large Telescope (VLT). An inaugural ceremony was held on 6 February at ESO headquarters in Garching to release the good news.

The active optics system relies on 75 servos that can slightly flex the 24-cm thick, 3.6-metre diameter primary mirror of the NTT. The servos are controlled by an image analyser, which looks at 'raw' star images to determine how the mirror shape should be adjusted to compensate for the varying distortions introduced by the passage of light through the Earth's atmosphere.

In good conditions, NTT can resolve star images down to 0.3 arc-seconds, a factor of three better than a conventional 3.6-metre at the La Silla site, in the Chilean Andes, typically achieves.

NTT's success is important for the development of the VLT, an array of four linked 8-metre telescopes to be built in Chile in the mid-1990s. VLT's mirrors will be thinner than NTT's, allowing more rapid adjustment of the mirror profile and therefore even higher resolution. Eventually, NTT will also be fitted with adaptive optics, a faster version of active optics (see *Nature* 341, 675; 1989).

In an inaugural observing run, done before the telescope had been fully optimized, NTT resolved stars down to 25th magnitude in ten minutes; ESO astronomer Richard West said that 27th-magnitude stars are expected to be resolved in a one-hour exposure.

NTT reveals the detailed structures of galaxies and globular star clusters with unprecedented clarity. A five-minute exposure on 3 December 1989 resolved individual stars in a globular cluster asso-

ciated with the Fornax dwarf galaxy, 700,000 light-years away, the first time such a distant cluster had been fully resolved (see Fig. 1). Another project will be a search for Cepheids, variable stars whose characteristic brightness changes allow their distance to be determined, in far-off galaxies.

Finding Cepheids is an essential step in estimating the Hubble constant, which measures the rate of expansion of the Universe.

On a night with relatively poor seeing conditions, the NTT was used to image Comet Austin, which is expected to become visible in the Northern Hemisphere by mid-April 1990. A hot spot was seen in the nucleus of the comet, even though it had not yet begun to develop a tail. This was the first time such a feature had been observed on a comet so far from the Sun. (see Fig. 2) Another innovation on NTT will be the use of a satellite links between La Silla and Garching, which will allow astronomers to direct the telescope without leaving Europe. This new system will allow flexibility in scheduling telescope time that was never possible when research teams had fixed stays in faraway Chile. The NTT is already a popular instrument: only a few observing slots are still available this year, after 1 October, and the deadline for proposals is 15 April.

JAPANESE ELECTION

US increases pressure on trade issues

Tokyo

THE comfortable win for Japan's ruling Liberal Democratic Party (LDP) in the general election on 18 February is expected to open the way for increased US pressure on Japan over science-related trade issues.

The United States had left several contentious trade issues to simmer while the LDP struggled to regain its foothold on power after a series of money and sex scandals brought a crushing defeat for the party in last year's upper house election. The United States clearly views the LDP as preferable to the Japan Socialist Party (JSP), which has traditionally opposed the US-Japan security treaty.

Among the issues that are likely to emerge are trade in satellites and supercomputers. The United States has been pressing the Japanese government to buy more US supercomputers for years but nearly all public sector orders still go to Japanese companies despite the fact that the US company Cray Research Inc. is the acknowledged leader in this field. Ironically, Japanese private companies such as Toyota and Toshiba are among Cray's best customers. But very few universities and government research institutes have Crays.

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

FIG. 2 Comet Austin — computer enhanced photo showing curves of equal brightness.

NTT has already inspired imitators. Italy is building a 3.5-metre telescope called Galileo that is modelled on NTT. (Italy and Switzerland financed a large proportion of NTT when they joined ESO and paid the entry fee in 1982.) Other groups from Australia, South Africa and the United States, as well as several groups from Europe, have expressed interest in purchasing the engineering knowhow for NTT.

Speaking at the inauguration ceremony, West German Research Minister Heinz Riesenhuber praised ESO for delivering NTT on time and within its budget of DM 24 million (\$14 million). He said that the same kind of efficiency will be necessary to ensure the successful completion of VLT.

Steven Dickman

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

FIG. 1 The Fornax dwarf galaxy — the first time a cluster so far away has been fully resolved.

Priority and the Raman effect

SIR—Sachi Sri Kantha¹ once again raises the question of priority in the discovery in 1928 of light scattering with changed frequency, called the Raman effect or combinational scattering of light.

Kantha attempts to belittle the part played by Landsberg and Mandelstam in the discovery of the new phenomenon, although it is widely known that they discovered the same phenomenon at the same time independently of Raman, gave it a correct explanation but published their results^{2,3} later than Raman and Krishnan^{4,5}.

A similar attempt to belittle the discovery of Landsberg and Mandelstam made necessary the publication of a note by the editors of the *Journal of Raman Spectroscopy*⁷. I also pointed out⁸ the full value and independence of Raman of the discovery made by Landsberg and Mandelstam.

In a speech⁹, Raman said that "the line spectrum of the new radiation was first seen on the 28 February 1928".

In Mandelstam's letter to O. D. Khvolson, we read: "We first noted the appearance of the new lines on February 21, 1928. On a negative from an experiment of February 23–24 (exposure time 15 hours) the new lines were clearly visible." The photographs made from this film have been published^{8,10}.

Landsberg and Mandelstam reported their discovery at the beginning of August 1928 at the sixth Congress of the Association of Russian Physicists. Twenty-one of the 400 participants were foreign scientists, among them Born, Brillouin, Darwin, Debye, Dirac, Phol, Pringsheim, Ph. Frank and Scheel. Accounts of the congress were published by Darwin in *Nature*¹¹ and by Born in *Naturwissenschaften*¹². Darwin wrote: "Perhaps the most interesting work is that of Prof. Ioffe . . . and that of Prof. Mandelstam and Landsberg. The latter described how they had independently discovered Raman phenomenon, the scattering of light with changed frequency."

Born wrote: "The effect discovered by Landsberg and Mandelstam in crystals is essentially identical to the effect observed by Raman and his colleague Krishnan in liquids. Russian physics can justly take pride in the fact that this important discovery was made by the Moscow researchers independently of the Indians and nearly simultaneously (February 20, 1928). This coincidence is one more demonstration of the international nature of our science, which now spans the entire world."

In the paper by A. Jayaraman and A. V. Ramdas¹³ devoted to the centenary of C. V. Raman, Indian physicists fairly wrote: "Really the Raman effect was independently discovered by Landsberg

and Mandelstam in calcite and quartz crystals".

By 1930 more than 100 papers of different authors had been devoted to the investigation of this new phenomenon. It was well known by scientists that Raman and Krishnan in Calcutta and Landsberg and Mandelstam in Moscow had simultaneously and independently discovered the same remarkable phenomenon.

Justice demanded that the 1930 Nobel prize for physics for this discovery should be awarded to Raman, Landsberg and Mandelstam. Regrettably, that did not happen.

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To publish or not to publish

SIR—In his leading article "Publicity without being damned" (*Nature* **343**, 113; 1990), John Maddox leaves the impression that *Physical Review Letters* may have done the scientific community a disservice by publishing, without comment, the extraordinary results obtained by two Japanese scientists on the weight of gyroscopes in motion. I am not so sure. It could be argued that it is better to let the scientific community puzzle it out and subject the data to experimental verification without attempts to belittle or prejudice the interpretation of experiments that the referees were unable to fault. *Nature* would almost certainly have emerged with more dignity from the "celebrated case in 1988" had it acted in the same way. Instead, it left a trail of unhappiness and discrimination that, in the final analysis, did not reflect much credit on anyone involved in the affair, although it may have provided some entertainment.

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Schrödinger in pre-war Germany

SIR—May I add some background information to P. W. Atkins' review of W. Moore's biography of Schrödinger (*Nature* **341**, 193–4; 1989) and its discussion by Popper (*Nature* **342**, 337; 1989)?

In the spring of 1933, while I was a student at the University of Berlin taking the course in theoretical physics given by Schrödinger, I visited him to discuss a possible topic for a thesis. During that conversation, or soon thereafter, we both decided to leave Germany; he because he was disgusted with the political situation and I for even weightier reasons.

During our meeting, Schrödinger recounted an anecdote that David Hilbert, the great Göttingen mathematician, had told him. During the First World War, in the winter of 1916, the rector of the university invited the professors to gather for an important announcement. Hilbert thought, aha, he will tell us that we will get a goose for Christmas; but no, the rector announced that unrestricted U-boat warfare had been declared. Hilbert concluded: "I realized then that they were quite mad", adding "One has to imitate them in every detail." (*"Man muss sie nachahmen bis ins kleinste."*)

After Austria was annexed by Germany in 1938 and Schrödinger felt trapped in Graz, he probably remembered Hilbert's advice when he wrote to the senate of the university. In this letter he appears to me to be clearly imitating the 'joyous' Nazi style.

MAURICE GOLDBABER

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Not so new

SIR—You recently published a note by Tania Ewing about an Australian 'invention', the tamper-proof passport (*Nature* **342**, 465; 1989). The fundamental method, however, of placing a picture on a passport by holographic means has been used here in Germany for our new ID-cards and passports for at least two years.

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Carbon tax

SIR—I wrote *nominal* not "normal carbon tax" in my letter (*Nature* **342**, 730; 1989). Indeed, the very thrust of my calculation argues against a carbon tax, for at any reasonable level, such a tax would have far less effect in engendering fuel switching or energy economy than direct fiscal and investment measures.

MAX K. WALLIS

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Between objectivity and subjectivity

Carl-Ivar Brändén and T. Alwyn Jones

Protein crystallography is an exacting trade, and the results may contain errors that are difficult to identify. It is the crystallographer's responsibility to make sure that incorrect protein structures do not reach the literature.

STRUCTURAL knowledge of biological macromolecules is essential for fully understanding their function as well as for changing that function by protein engineering or drug design. There are two powerful, complementary methods to obtain the structure of large molecules — X-ray crystallography and two-dimensional nuclear magnetic resonance spectroscopy. For good reasons, the biological community has regarded X-ray structures as gospels of truth because X-ray diffraction data in principle contain sufficient information to extract the correct structure. This faith has been shaken by studies¹⁻⁵ showing that there are serious errors in several recently published X-ray structures⁶⁻¹⁰; among the structures concerned are an electron-transport molecule, ferredoxin^{1,6}, the glycolytic enzyme enolase^{2,7}, the small subunit of the CO₂-fixing enzyme Rubisco^{3,8} (ribulose-1,5-bisphosphate carboxylase), the HIV-proteinase^{4,9}, and the Ha-ras oncogene product p21 (refs 5, 10). In view of the rapid evolution of macromolecular crystallography, one may well wonder why this situation has arisen. Here we explain some of the problems that crystallographers face in interpreting their data, and argue for the need to devise better checks on their results.

Technical developments. In the past ten years there has been a minor revolution in structural molecular biology. Developments in gene technology now allow expression of the large amounts of protein (what the cloner likes to call 'gene product') usually needed for structural work. This improves the chance of obtaining crystals suitable for structure determination simply by allowing us to try a much larger variety of growth conditions. Crystallization robots are now used in a number of laboratories in the hope of speeding up this process. Other technical advances have had an even more profound influence. Collection of diffraction data has been revolutionized by new X-ray detectors and X-ray sources. Electronic area detectors of various types allow the rapid collection of diffraction data in a form immediately suited for computer processing. High-energy electron (or positron) synchrotrons and storage rings produce very intense X-ray radiation, thereby allowing the rapid collection of data and increasing crystal lifetime. Bombarding a crystal with X-rays always

destroys the crystallinity of the sample. But because the process is time-dependent, short and intense exposure gives better results (and also allows the use of smaller crystals).

Many aspects of treating diffraction data before arriving at the 'final' model are computer intensive, and crystallographers have benefited enormously from the 'silicon revolution' in which the price: performance ratio of computers is halved every 18 months or so. Developments in computer graphics have been almost as impressive.

Together, these improvements have allowed us to take on and solve many incredibly complex structural problems. But at a price.

The crystallographic problem. The basic difficulty of protein crystallography remains unchanged — the problem of phases. In diffraction experiments we are able to collect only part of the data needed to reconstruct the repeating unit within the crystal. Diffraction data can be considered as complex numbers where we can measure only the amplitude but not the phase of each number. In small-molecule crystallography this problem has been solved by so-called direct methods (recognized by the award of a Nobel prize in chemistry to Karle and Hauptman). Where brains fail, protein crystallographers have stayed at the laboratory bench using techniques pioneered by Perutz, Kendrew and co-workers to circumvent the phase problem. This method, called multiple isomorphous replacement (MIR), requires the introduction of new X-ray scatterers into the crystal unit cell. These additions should be heavy atoms (so that their presence can be felt in the diffraction process); there should not be too many of them (so that we can find them); and they should not change the structure of the molecule or of the crystal cell. This final requirement is often incompatible with the others.

If a number of heavy-atom derivatives can be obtained, it is then possible to calculate an electron-density map showing the repeating unit of the crystal. The map is a three-dimensional set of numbers that has to be interpreted as a polypeptide chain of a particular amino-acid sequence. But although it is compiled from objective information, the map contains numerous errors; its interpretation remains to some extent subjective. It should be emphasized

that there is a fine line between an uninterpretable map and one from which a reasonable model can be built. Errors in the model introduced by the crystallographer's interpretation depend on the map's quality, which in turn depends on several factors.

(1) Accuracy of the diffraction measurements — small differences between large numbers are used to obtain the phases.

(2) The number and usefulness of the heavy-atom derivatives — metal atoms that bind isomorphously to a few different sites are ideal.

(3) Resolution of the diffraction data, which refers to how well ordered the crystals are and directly influences the image that can be produced. Native crystals (that is, those with no heavy atoms added) often have better quality diffraction than the derivatives, limiting the quality of the initial map but providing hope for ultimately determining a high-quality model. At 4 Å resolution there is little side-chain detail. At 3 Å it should be possible to decide between the density for an alanine side chain and a leucine; at 2 Å, between a leucine and an isoleucine; and at 1 Å one sees atoms as balls of density (Fig. 1; see over). As far as we are aware, the lowest resolution at which an essentially correct model has been built is for satellite tobacco necrosis virus¹¹ at ~3.7 Å. In this study use was made of the high symmetry of the virus capsid to produce phases with essentially no errors. This is an unusual case and normally phase errors are much larger for the higher resolution data. In normal studies we aim for an MIR map based on phases determined to ~3 Å resolution or better.

Building a model. Building an initial model is a two-stage process. First, one has to decide how the polypeptide chain weaves its way through the electron-density map. The resulting chain trace constitutes a hypothesis, where one tries to match the density to the sequence of the polypeptide. An initial model is then built to give the best fit of the atoms to electron density. Both processes use computer graphics to present the data and manipulate the model building.

Tracing the chain has yet to be automated and is often one of the most difficult steps in solving a new structure. In practice the process requires recognizing some part of the sequence in the map by the

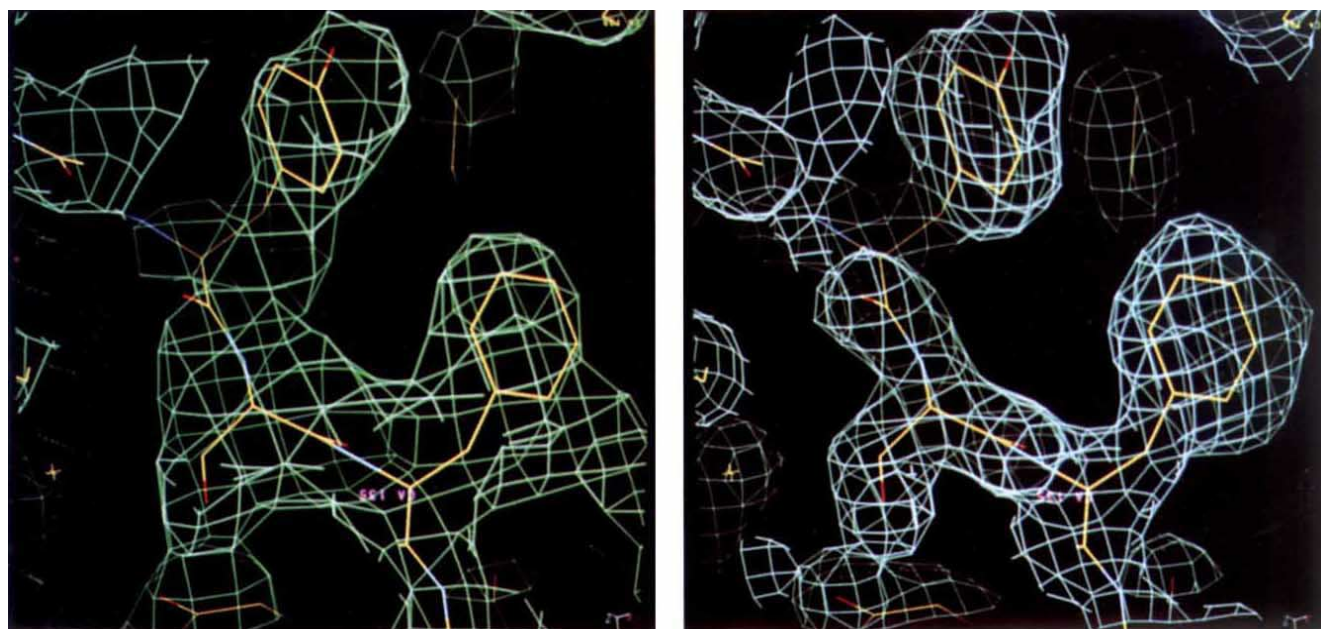


FIG. 1 Views of part of the electron density for the retinol-binding protein with superimposed final model (left) after model refinement to 2.0 Å resolution, and (right) from the MIR map at 3.1 Å resolution.

shape of the electron density. One then tries to extend this alignment in both directions in the sequence until the quality of the map or the matching to the sequence breaks down. This sounds easy but it is not; a map showing continuous density from N terminus to C terminus is rare. More usually one produces a number of matches of discontinuous regions of the sequence that may initially account for a small fraction of the molecule and may be internally inconsistent. For example, two hypothetical segments may come together representing entirely different parts of the sequence and even be in different directions. Chain direction can also be determined by recognizing bumps in the density representing carbonyl oxygens, but this requires high-resolution data.

At this stage one needs an overview of how the chain trace is developing, and also a detailed view to identify where we are in the sequence. Three-dimensional contoured nets¹² are used to portray a detailed view of the density. An overview can be produced with a skeletonized representation of the map¹³ (analogous to a thread of string passing through the main features of the density), which is easy to change by removing incorrect connections or adding new ones and which can be coloured to illustrate the current state of the chain trace¹⁴.

At any stage in this process, the chain trace has to relate to what is known about protein structure. The α -helices and β -strands are easily recognizable because of their distinct pattern of adjacent side chains. α -helices are invariably right-handed; it was the presence of left-handed α -helical features in the electron-density map of ferredoxin that provided the essential clue¹ for the cause of error⁶ in that

structure.

The chain trace must also relate to chemical and physical information about the protein. Knowing the positions of the heavy atoms used to determine the structure is helpful. These metal ions should have proper protein ligands¹⁵ — mercury atoms usually bind to cysteine residues, platinum atoms to methionine residues and the uranyl group to oxygen ligands. If endogenous metal ions are present in the protein they should also have proper protein ligands which are usually conserved in homologous proteins from other species. Important active-site residues should also be conserved. Deletions and insertions within a family of related proteins should occur in loop regions and not in the middle of α -helices or β -strands.

Correct connections of the α -helices and β -strands are usually the most difficult parts of map interpretation. The loop regions that connect these secondary-structure elements are on the outside of the molecule and are frequently more flexible; in consequence their electron densities are weaker and more distorted. Errors in such connectivity occurred in the incorrect solution of the structures of both Rubisco⁸ and p21 (ref. 10).

The α -helices and β -strands combine to form domain structures with specific folding patterns, some of which occur more frequently than others. So it can be helpful in map interpretation to see whether simple reconstructions result in a known, common fold.

One of the most frequently observed domain structures is the α/β barrel which comprises eight parallel β -strands arranged like the staves of a barrel and surrounded by eight α -helices. In the early days of protein crystallography, Jane

Richardson¹⁶ suggested that the preliminary model of the enzyme KDPG-aldolase¹⁷ could be reconnected to produce such a regular α/β barrel. High-resolution work confirmed this proposal and the structure was corrected¹⁸. On the other hand, it can be very dangerous to force a model to conform to a known domain structure. The enolase structure was first interpreted as a regular α/β barrel⁷, but further refinement established that some connections were wrong² and that one of the β -strands was in fact antiparallel to the other seven strands.

Having obtained a full trace (or perhaps a partial trace to check how sure one really is) various methods have been developed to build a model. We believe the use of databases offers a number of advantages. Fragments from a database of highly refined structures can be extracted to best fit the skeleton¹⁴. This forces the use of stereochemically correct pieces of main chain¹⁴. Most of the amino acids adopt only a few preferred side-chain conformations, called rotamers¹⁹. For example, 67 per cent of all valine residues adopt a single conformation. We therefore build our initial models fully automatically from the C α trace using the main-chain and side-chain databases.

Removal of errors in the initial model.

The final model coming from the MIR map will contain errors. Provided the native crystals diffract to high enough resolution, most of them can be removed by crystallographic refinement of the model. In this process, the model is changed to improve the fit of the structure factor amplitudes calculated from the model ($|F_c|$) with the observed amplitudes ($|F_o|$). This process is computationally

intensive. The goodness of fit is expressed as the R factor ($R = \sum |F_o| - |F_c| / \sum |F_o|$) where the sum is taken over the diffraction measurements. The results are then reviewed at the graphics terminal, and the model changed accordingly. The model is then subjected to continued refinement until no further improvement is obtained. The refinement techniques have been based on least squares methods²⁰, but recently methods based on molecular dynamics algorithms²¹ have become widely used. These are even more computationally expensive.

The errors vary in severity:

(1) The model may be totally wrong (although that is unlikely if one has a sequence) because the whole chain trace is incorrect; individual secondary-structure elements may be correctly identified, but their directions may be wrong. Such a trace would inevitably contain incorrect connections between secondary-structure elements. The error may also be due to a more basic mistake such as wrong space group⁶.

(2) In multi-subunit molecules, one subunit could be totally wrong⁸. Such a structure can only be corrected by returning to the initial MIR map. In this case, combining the MIR phase information with phases calculated from the correct part of the model may provide the basis for a better map.

(3) Main-chain connectivity may be partly wrong^{7,9,10,22}, which is the most common serious error and is usually the result of incorrect connections between secondary-structure elements. Such errors can sometimes be identified during refinement of high-resolution data and be corrected. The refinement of the incorrect enolase model stalled at an R value of 26.6 per cent for data to 2.25-Å resolution. The model was then revised and the correct structure easily refined to an R factor of 20.2 per cent⁷. In p21 and HIV protease, connectivity errors were not recognized during refinement and became apparent only when independent structure determinations were carried out. In both these structures, the incorrect models could be refined to R factors of ~25 per cent.

(4) Sequence out of register with the density over a small part of the structure. This is very common and should be correctable during refinement.

(5) Locally misbehaving model — some parts of the main chain may just be badly built and be beyond the radius of convergence of the refinement.

(6) Incorrect side-chain conformation. Once the major flaws have been corrected, these errors may be easier to spot in the new maps. If the correction is outside the radius of convergence of the refinement algorithm, the model may still be pushed into some distorted conformation mimicking the correct one. At each refitting stage we compare side-chain confor-

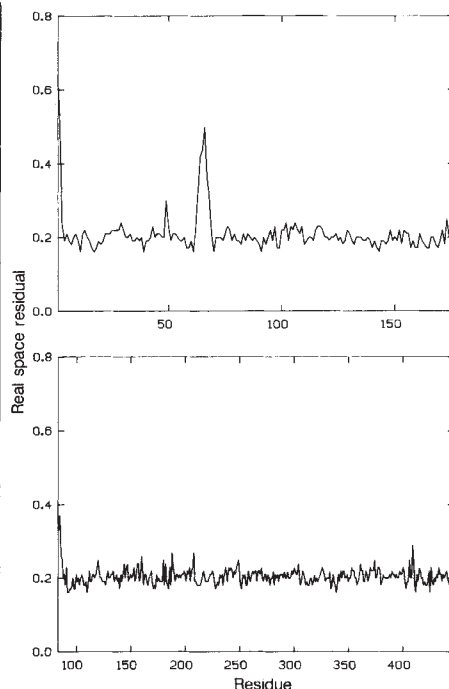


FIG. 2 The main-chain real-space R factor for (top) retinol-binding protein; and (bottom) cellobiohydrolase II. For the main-chain atoms of each residue the function calculates $\sum |e_o - e_c| / \sum |e_o + e_c|$ on the grid of the observed density (e_o), for all grid points contained in the map calculated from the atoms (e_c). This function can define the fit of the whole residue, the main chain or the side chain. It can unambiguously show the problem areas in a model. The top figure shows clearly a localized, poorly fitting section of the chain. The proteins have crystallographic R factors to 2.0 Å resolution of 18 and 15 per cent, respectively.

matings with the rotamer database¹⁹.

(7) Main-chain conformational errors (peptide flips). Although Cα atoms of neighbouring residues may be correctly positioned, the carbonyl oxygen of the linking peptide may be pointing in the wrong direction. This should be corrected during the refinement. We have found that using the database to build models reduces this problem. At each refitting stage we check the deviation of carbonyl oxygens from those of the best-fitting fragments in the database²³.

Refinement is a gradual process; as errors are located and corrected it becomes easier to correct remaining errors.

Assessment of the final model. There are a number of general points concerning assessment of reliability of a structure determination.

(1) The better the MIR map, the better the initial model, the easier is the refinement and the more reliable is the structure.

(2) The higher the resolution the better. Unfortunately, not all crystals diffract as well as we would like.

(3) The quoted R factor has to be viewed carefully for a number of reasons.

It is affected by the choice of low-resolution cutoff and by the removal of weak reflections. It is also insensitive to errors in main-chain connectivity. Alarm bells should ring if the refinement stalls at an R value of about 25 per cent for high-resolution data. We advocate the use of a real-space R factor²³ that shows, on a residue-by-residue basis, how good the fit is between the model and the map (Fig. 2).

(4) The final model should be tightly restrained to standard bond lengths, bond angles and fixed dihedral angles (reasonable values are 0.02 Å, 2° and 2°, although each refinement program has its own fingerprint).

(5) Main-chain dihedral angles should conform to the 'allowed' regions of the Ramachandran plot.

(6) The number of water molecules added during refinement should be realistic. For high-resolution structures a reasonable value is one water molecule for each residue, and these molecules should have plausible hydrogen bonds.

Protein crystallography is a highly competitive field where the same or similar structures are being worked on in a number of different laboratories. Although this may result in an urge to publish quickly and prematurely, it is the responsibility of crystallographers to check their preliminary models carefully before rushing into print. Journals must insist on the publication of enough data for crystallographers to convince the reader that they have a correct structure, and the readership should be sophisticated enough to judge the quality of the data. We strongly object to publication of structural work where authors supply a minimum amount of detail in the form of a cartoon. □

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Sellafield makes news again

Britain's uranium reprocessing plant is making waves again, not now because of its releases of radioactivity to the environment, but because a relationship between parental exposure and childhood leukaemia has been demonstrated.

FANCIFUL though it may seem, the recognition of an association between the exposure of potential fathers to radiation and the chance that their children will develop early leukaemia or lymphomas will be a relief for many managers of the nuclear industry, now mostly in any case (and for the time being) in limbo. Better the devil you know will be the principle.

For the past decade, there has been persistent anxiety about the reasons why two of the most conspicuous clusters of early-onset leukaemia and lymphoma in Britain should be near the nuclear plants at Sellafield and Dounreay (in the north of Scotland). Releases of radiation from the plants are well-monitored, and are unambiguously too small to account for the recorded cases of death among young people by the induction of mutations in somatic cells. In any case, several investigations have failed to discover why young people should have been particularly at risk over the past three and a half decades.

So the conventional explanation has been that the clustering phenomenon must be a matter of chance as, numerically, it could still be. Professor Martin Gardner and his colleagues are at pains to emphasize in their report (see page 679) that a statistical association does not prove a causal relationship. Indeed, their data show that the relative risk of contracting early leukaemia or non-Hodgkin's lymphoma is as great among the children of iron and steelworkers in west Cumbria as among the those of male workers at the Sellafield nuclear plant. (The relative risk is measured with reference to a matched control group of 16, drawn for each case from the local and regional population.)

Yet the data also include some straws in the wind that, while not conclusive, will be taken as suggestive. In the whole sample, there seem to have been ten children or young people whose fathers could be identified as having worked at the Sellafield plant; their records were complete enough for their accumulated radiation doses to be estimated in the period up until conception. What emerges is that the relative risk increases with the total dose.

Of course, the numbers are so small that no conclusions can be logically drawn from them, but the mere fact that the relative risk increases with the dose must strengthen the suspicion that pre-conception exposure to radiation is a cause of early leukaemia and lymphoma. And that is the sense in which the new study will be a relief: if the excess of leukaemia around

the reprocessing plant at Sellafield is not a statistical artefact, it is better to have an inkling of its cause than to have to believe that it comes about by magic.

But that is only part of the problem with which Sellafield must now contend. The new study, while suggestive, is dauntingly inconclusive. From the data gathered by Gardner and his colleagues, it is not even possible to say ("guess" would be more appropriate) whether the radiation exposure of fathers-to-be that matters is the whole accumulated dose or merely that accumulated in the few months occupied by human spermatogenesis immediately preceding conception. Yet this question will be crucial for the sensible management of radiation exposure. Men whose wives planned to become pregnant might then, for example, be taken off work entailing high exposure.

Statistically, there seems no easy way of tackling that problem. It would help if the study now described, in which data have been gathered from public records (at the Office of Population and Census and British Nuclear Fuels Ltd (BNFL), with appropriate safeguards) supplemented by questionnaires, could be made complete. That way, for example, it would be possible to include in the study more than the ten cases of leukaemia or lymphoma now contributing to the data on parental occupation. But that would require that the parents should be traced and questioned face to face, which would entail a breach of research ethics hardly justifiable by the statistical benefit that would arise.

It is just conceivable that something might be learned from a comparison of the relative risks now discovered at Sellafield with those obtaining for the whole of the United Kingdom. For if radiation dose, accumulated either over a few months or a lifetime in advance of conceiving children, is a cause of leukaemia at Sellafield, so must natural radiation cause early leukaemia elsewhere. It would be interesting to know whether the simple-minded relationship between accumulated radiation dose and leukaemia incidence suggested by the Sellafield data compares in any way in magnitude with the natural occurrence of leukaemia, at least some of which must be induced by the same hypothetical mechanism.

There is probably more hope of pinning down whatever mechanism there may be with new techniques in cytogenetics and molecular biology. If radiation damage to a potential father can lead to the devel-

opment of leukaemia in a child, the damage must consist of damage to some chromosome. Visible breaks in chromosomes in, for example, circulating lymphocytes are well-known consequences of radiation exposure; their frequency increases with dose, and may be diagnostic of the degree of exposure.

Less is known of the frequency of abnormal chromosomes in gametes, while it is probable that only some of the abnormalities leading to leukaemia have as yet been identified. (At least some of them are translocations, and so not detectable by mere counting.) Yet investigations among people who have been occupationally exposed to radiation, sometimes *post mortem* and not necessarily at Sellafield, might make it possible to tell how the burden of damage is distributed between spermatozoa and the stem cells from which they spring, in the spermatogonia. If the former but not the latter, radiation exposure might be dangerous only in the few months spent on the formation of spermatozoa from stem cells. Otherwise, it would be more probable that the lifetime accumulation of radiation, natural and otherwise, is what matters.

That would be managerially inconvenient because of the relationship between the operators of nuclear plants and those who work for them. Sellafield, as it happens, has benefited enormously from the loyalty of its workforce even during the period when the plant seemed careless of its reputation for good management. The people there have stoically accepted the well-documented risk that radiation exposure can cause cancer of various kinds, but at a low frequency. But if it were shown that radiation exposure can cause leukaemia among children, even at a lower frequency hardly distinguishable from that expected naturally, the reaction would probably be out of proportion to the numerical frequencies, whatever they are.

The moral is that occupational exposure will have to be reduced. BNFL, the operator at Sellafield, boasts of solid progress in the past few years in reducing the doses to which people are exposed. New processing plants have helped. But it is too easy to forget that occupational exposures may exceed by two orders of magnitude those to which people are naturally exposed. For short times, that may be tolerable because inconspicuous, but in the long haul it must catch up with people.

John Maddox

Exposing molecular motions

Ian W. M. Smith

ONE amusing characteristic of chemists is their tendency to talk as if they can really 'see' atoms and molecules as clearly as objects in the everyday world. To do this in reality implies first the ability to observe individual atoms and molecules and second the power to monitor their extraordinarily rapid motions. The first requirement can now be satisfied for the essentially static atoms in a solid surface by the technique of field-ionization spectroscopy. The second, which is crucial, since energy and motion are central to the process of chemical reaction, is now becoming possible via spectroscopic techniques using laser pulses lasting only tens of femtoseconds ($1 \text{ fs} = 10^{-15} \text{ s}$), as illustrated on page 737 of this issue¹.

The requirement for such extraordinarily short bursts of light results from the extremely rapid motions of atoms and molecules and is entirely analogous to that in conventional photography if one wishes to obtain a sharply defined image of a moving body. A photograph taken of Linford Christie running at 10 metres per second will have a definition of 1 cm or better only if an exposure (or flash) time of 1 millisecond or less is used. Similarly, to obtain clearly defined spectroscopic 'pictures' of molecular vibrations, or of the relative motion of molecular fragments as molecules dissociate following

photofragmentation of a chemical bond, requires flashes of light shorter than the times characteristic of these motions — which are typically in the range 10 fs to 1 ps (10^{-12} s).

Experiments such as these are being pioneered in the laboratories of Ahmed Zewail. The characteristics of the laser pulses used are determined by Heisenberg's uncertainty principle. As a laser pulse is made shorter, and hence the time resolution based on the use of such pulses is improved, the light contains a wider range of frequencies so that spectral resolution is lost. Thus a 100 fs laser pulse has a wavenumber bandwidth of about 150 cm^{-1} . A consequence of this is that when molecules are excited with such a short pulse, they are necessarily prepared with a spread of energies. But because both the preparation and observation of molecules are coherent processes, the time resolution is not degraded and motions can be 'clocked' with a precision similar to the duration of the laser pulses.

Experiments using femtosecond spectroscopy have now been used to probe an exciting variety of molecular processes. In this issue¹, Zewail and colleagues report the direct observation of molecular vibrations and rotations in I_2 . Generally, the periods associated with these motions are inferred by using the equations of quantum mechanics to relate the pattern of stationary-state energy levels to the fundamental properties of the molecules, in particular the frequency or momentum of the motions. The time-resolved approach, using stroboscopic snapshots, is quite different.

For example, to investigate rotation, one short pulse of polarized laser light is used to electronically excite I_2 molecules which are in a range of rotational levels but which have the same spatial orientation. A second, similarly short and similarly polarized laser pulse is used to excite the prepared molecules to a still higher state from which fluorescence occurs, and the intensity of this emission is observed. As the delay time between the two pulses is increased from zero, one first sees a decrease in the laser-induced fluorescence (LIF) signal as molecules rotate away, at different speeds, from their initial favoured orientation. However, the frequencies of rotation of molecules in different states are integral multiples of the slowest moving molecules. Consequently, at the instant when these slowest molecules have executed a single rotation and returned to the originally prepared orientation, all other molecules have undergone an integral number of rotations, the molecules are realigned and the LIF signal at this

delay shows a clear peak in intensity. The interval for such rotational recurrences precisely measures the fundamental rotational period.

Related measurements, but subtly different and on a much shorter timescale, are reported for the I_2 vibration. The differences arise because the vibrational motion is much less harmonic than the rotations. The Fourier transform of the observed signal shows clear evidence that more than one frequency results from the preparation of I_2 in more than one vibrational state, each having its own characteristic frequency.

Although it is fascinating to be able to observe directly the periodic motions of molecules in stable states, even more important from a chemical standpoint is the power of femtosecond spectroscopy to follow chemical changes as bonds break and new species form. Three examples exemplify what has been achieved. The first is a case of direct photolysis^{2,3} and was the subject of an earlier News and Views article⁴. LIF was observed from the CN radical during its formation from photofragmentation of ICN. With the frequency of the probe laser tuned to be resonant with a value in the spectrum of free CN, there was a delay of approximately 200 fs before the LIF signal reached its full intensity. This is due to the fact that the close proximity of the separating I atom perturbs the CN spectrum. As a consequence, by tuning the probe laser to different frequencies, snapshots of fragmenting molecules can be obtained at different $\text{I} \cdots \text{CN}$ spacings as they evolve to entirely separate $\text{I} + \text{CN}$ species. Measurements of both kinds determine how long the bond-breaking process takes and allows

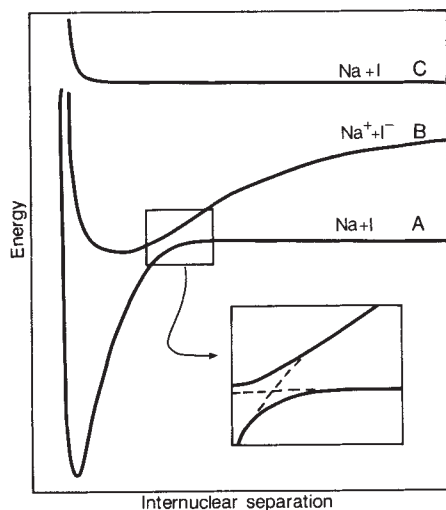


FIG. 1 A schematic of the potential-energy curves, depicting the strength of interaction between the constituent atoms, for the three relevant states of NaI. Molecules are excited from the strongly bound ground state (A) to state B. Their progress along the separation coordinate is monitored using femtosecond 'probe' laser pulses that cause excitation to curve C from which fluorescence occurs. The inset shows the region where there is an 'avoided' crossing between the diabatic curves (dashed lines), with minimum rearrangement of molecular electron distribution) and adiabatic curves (unbroken lines).

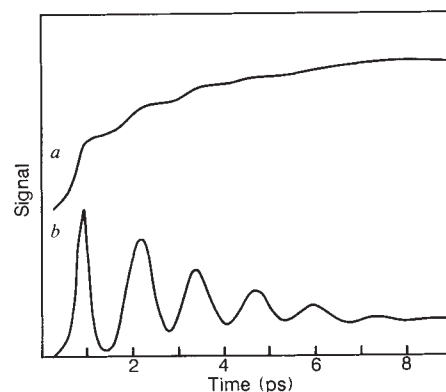


FIG. 2 Laser-induced fluorescence signals obtained following femtosecond excitation of NaI when the laser is tuned (a) into resonance with the spectral lines of free Na atoms, and (b) away from resonance with this transition. Curve a rises in a series of steps as NaI systems 'leak' through the avoided crossing with a probability of less than one during each vibration. Curve b monitors the passage of bound NaI through a particular internuclear separation. The peaks broaden because of anharmonicity and are damped because of the partial dissociation during each vibration.

the shape of the dissociative potential between I and CN to be deduced.

More recent experiments have probed the dynamics of 'obstructed' photodissociation and sequential photodissociation. The first process has been studied in sodium iodide⁵. In their lowest electronic states, alkali halides are strongly ionic (almost Na^+I^-) but their lowest dissociation limit yields neutral atoms, $\text{Na} + \text{I}$. Consequently, the potential curves for ionic and covalent states undergo an 'avoided crossing', as shown in Fig. 1. Every time the system passes through this point, there is a chance it 'jumps the tracks'. The experiments using femtosecond spectroscopy, like those on ICN, can monitor the existence of the system at different internuclear separations. With the probe laser tuned to the absorption frequency of free Na atoms, the signal rises in a series of steps as is shown, in idealized form, in Fig. 2a. Individual steps correspond to 'leakage' of the system out to dissociation on the covalent curve each time it passes through the avoided crossing. Figure 2b shows a signal obtained with the probe laser tuned off-resonance. These signals are not unlike the periodic signals obtained in the case of I_2 and associated with vibration of the molecule. But, in this case, as well as the peaks broadening owing to anharmonicity, the signal is damped as a fraction of excited NaI dissociates each time it passes through the avoided crossing.

A final example to demonstrate the power of rapid time-resolved spectroscopy is provided by experiments on the photofragmentation of $\text{C}_2\text{F}_4\text{I}_2$, using 'only' picosecond laser pulses⁶. Absorption of a single ultraviolet photon causes two bonds to break yielding C_2F_4 and two iodine atoms. The concentrations of atoms in their electronic ground state (I) and in an excited state (I^*) can be monitored independently using two quite different probe-laser wavelengths. As Fig. 3 shows,

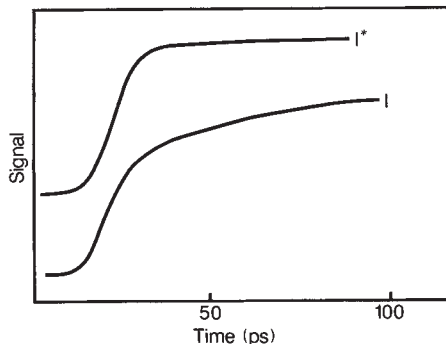


FIG. 3 Signals obtained from ground-state (I) and excited (I^*) iodine atoms following excitation of $\text{C}_2\text{F}_4\text{I}_2$ with ultraviolet picosecond laser pulses. The rise on the I^* signal reflects the pulse width of the photolysing laser. The I signal has a similarly rapid component, as the primary direct photodissociation yields a mixture of I and I^* , and a much slower component arising from unimolecular dissociation of $\text{C}_2\text{F}_4\text{I}$ radicals.

fragmentation occurs in two successive steps. The first occurs in less than a picosecond and yields mainly but not exclusively I^* atoms in a direct photodissociative process which can form the basis for iodine atom laser action. The second step is much slower, taking 30–150 ps depending on the energy of the initially absorbed photon, and it produces only I atoms. It is clear that this secondary process is akin to unimolecular thermal dissociation. For this dissociation to occur, internal energy left in the $\text{C}_2\text{F}_4\text{I}$ radical following the initial dissociation must have time to accumulate in the C–I bond before it can break.

Initial doubts that femtosecond spectroscopy might be applied to only a limited

range of molecular systems and processes are being assuaged. The applications described here demonstrate clearly that the technique is going to have a wide-ranging impact on chemistry. □

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MOLECULAR GENETICS

A gene for Wilms tumour?

Uta Francke

WILMS tumour (nephroblastoma) is a common renal tumour of early childhood; like retinoblastoma, it is made up of incompletely differentiated tissue. Both types of tumour occur unilaterally or bilaterally, with single or multiple foci, and in a sporadic or inherited fashion. Inheritance of a predisposing gene or presence of a specific constitutional chromosomal deletion is associated with multiple tumours and earlier onset, whereas sporadic tumours are mostly singular and are diagnosed later. The deletions, in band 13q14.1 for retinoblastoma and in 11p13 for Wilms tumour (reviewed in ref. 1), have enabled researchers to pinpoint the sites of the loci responsible and successfully to clone the retinoblastoma gene^{2,3}. Using the same general strategy that has led to the cloning of the genes for Duchenne muscular dystrophy, X-linked chronic granulomatous disease and retinoblastoma, Gessler *et al.*⁴, as reported on page 774 of this issue, have now isolated a candidate gene for the Wilms-tumour locus in 11p13.

The 11p13 region, first implicated in Wilms-tumour patients with constitutional deletions, was further subdivided by physical mapping techniques using additional chromosomal deletion and translocation breakpoints⁵, large-scale pulsed-field gel electrophoresis restriction maps as well as chromosome walking and jumping techniques. Gessler *et al.*⁴ have isolated four CpG-rich sequences or 'CpG islands', which often surround promoters of so-called housekeeping genes, within 650 kilobases (kb) of band 11p13 and have probed flanking sequences for cross-species conservation and for expression in normal kidney, in Wilms-tumour tissue and in other cell types. Two complementary DNA clones associated with CpG islands were isolated; one of these was

expressed ubiquitously, whereas expression of the other (called LK15) was limited to fetal kidney where it hybridized to a 2.9-kb messenger RNA. The combined sequence of LK15 cDNA clones is nearly complete with its 5' end within 4 kb of the CpG island. The predicted amino-acid sequence contains four tandem zinc-finger motifs known to be associated with transcription factors. The zinc-finger domain contains the only detectable similarity to other known genes, in particular the *EGR2* (early growth response) gene. The LK15 gene spreads over roughly 60 kb of genomic DNA and maps to a region between 60 and 170 kb in size that is homogeneously deleted in the two independent Wilms-tumour cell lines PER and 8A.

The gene, although incomplete as yet, is therefore a candidate for the Wilms-tumour locus. The authors are cautious not to say so, however. Their reasons for caution are: first, there could be one or two more genes in the region homogeneously deleted in both Wilms-tumour lines that they have not yet found; second, the LK15 gene was neither absent nor rearranged in 63 out of 65 Wilms-tumour samples examined, the exceptions being PER and 8A; and, third, a transcript of normal size was found in 12 tumours examined.

The main motivation for the rush to publish their results, even in the face of these uncertainties, is likely to be found in the intense competition in this field. As was highlighted in a recent report in *Science*⁶, Katherine M. Call and colleagues have described, at the annual meeting of the American Society of Human Genetics in Baltimore in November 1989, the independent isolation of what appears to be the same gene⁷. A sequence isolated from a cosmid that mapped in the Wilms-tumour interval of band 11p13 (ref. 5), was found to cross-hybridize to DNA

from different species and to be homozygously deleted in yet a third Wilms-tumour cell line (WiT-13). As published in *Cell* this week⁸, their 2.3-kb cDNA sequence is 210 amino acids shorter than that of Gessler *et al.* and also contains the 3' untranslated region. It starts at nucleotide 633 of Gessler *et al.*'s sequence and is missing 17 amino acids (376–392) which could be due to alternative splicing or to technical problems. A 3-kb mRNA was found in kidney and spleen and in the human erythroleukaemia cell line K562 and the T-cell lymphoma line CEM. The transcript was absent in numerous other tumour cell lines. As reported by Call at the meeting, transcripts of normal size and abundance were found in all of 12 sporadic Wilms-tumour tissues studied. (By contrast, the retinoblastoma gene is either not at all, or aberrantly expressed in the majority of retinoblastomas.) Notwithstanding this finding, the authors of the *Cell* paper⁸ conclude that the genetic localization, the tissue-specific expression and the function predicted from the sequence are evidence enough to suggest that they have cloned the elusive Wilms-tumour locus in 11p13.

This conclusion can only be evaluated if one considers the striking differences between retinoblastoma and Wilms tumour. For retinoblastoma there is strong evidence that a single gene is involved. It was originally suggested that this gene was located on chromosome 13, band q14, based on the identification of individuals suffering from heterozygous constitutional deletions, mental retardation and bilateral multifocal retinoblastomas. Studies of tumour tissue have revealed structural chromosome changes or loss of heterozygosity at the same site; this is consistent with Knudson's two-hit hypothesis¹, where the second hit would involve the other allele at the retinoblastoma locus. Family linkage studies have mapped the highly penetrant autosomal dominant retinoblastoma mutation to exactly the same band. The gene responsible for retinoblastoma (*RB*) was isolated independently by several groups based on its location, its absence in cell lines from patients and from tumours with small deletions, its expression in most tissues including normal retina, its inactivation in retinoblastoma tumours, and its ability to suppress tumorigenesis when transferred into retinoblastoma cell lines^{2,3}. It encodes a phosphorylated nuclear protein that is not likely to bind DNA directly and has no amino-acid similarity with the LK15 product. The *RB* gene has acquired the designation of a 'tumour-suppressor gene', especially as lack of expression was also found in tumours other than retinoblastoma.

By analogy, a Wilms-tumour gene was assigned to chromosome 11p13 by the study of individuals with constitutional deletions who exhibited aniridia (congen-

Experimentation by candle light

An Experiment on a Bird in the Air Pump, by Joseph Wright (1734–97), illustrates a favourite demonstration of travelling science lecturers in the eighteenth century. The painting is included in an exhibition

hand, raised to an air valve, could end the suffering at any moment. But a decayed skull in the glass jar, masking the candle, symbolizes the presence of death. Wright's reputation as an artist of

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

of Wright's work, now showing in London before moving on to Paris and New York.

The vital need for air was revealed by subjecting a small animal to a vacuum; air was removed from the glass bulb by a hand pump, seen here beneath the lecturer's right arm. The use of a white cockatoo can be attributed to artistic licence, the white plumage catching the flickering candle light, as rats and sparrows would usually be preferred.

The picture is notable for the range of expressions depicted: the horror of the small girls; the dispassionate interest of the seated spectator to the left, timing the experiment with pocket watch; and the exchange of glances between the lovers. The focus of the scene, though, is the fate of the struggling bird: the lecturer's left

**Wright of Derby* is at the Tate Gallery, London (until 22 April). It will subsequently appear at the Grand Palais, Paris (17 May – 23 July) and the Metropolitan Museum of Art, New York (6 September – 2 December).

scientific and industrial subjects rests on a handful of paintings — all included in the exhibition — especially *The Air Pump*, shown here, and *A Philosopher giving a Lecture upon the Orrery*. The details of the scientific equipment itself were evidently one source of fascination. The inclusion in the picture by the girl's arm of the two halves of a Magdeburg sphere, which can be clenched together by a vacuum, suggests that the present experiment is the last in a lecture on pneumatics.

Wright's circle of friends included many members of the Lunar Society, a group of provincial scientists and industrialists which met at each new moon. Among them were Erasmus Darwin (grandfather of Charles), John Whitehurst, Josiah Wedgwood and Richard Arkwright (inventor of the spinning jenny), all of whom probably stimulated Wright in his choice of subjects. □

ital absence of the iris of both eyes), genito-urinary abnormalities in males, mental retardation and a tendency to develop Wilms tumour. This condition, termed 'WAGR (Wilms tumour/aniridia genitourinary/retardation) complex', was the first example of an autosomal micro-deletion that removes more than one locus and results in the combination of a number of traits that are otherwise known as single-gene, dominant disorders. A mendelian dominant gene for aniridia has been independently mapped to 11p13 (ref. 9).

Cytogenetic studies of Wilms-tumour tissues have revealed several cases of deletions or translocations at 11p13 involving one or both chromosomes 11. DNA marker studies looking for loss of heterozygosity in Wilms tumours have focused, therefore, on polymorphic loci on 11p. Surprisingly, the most consistent loss was observed for markers in band 11p15, ten or more megabases distal to the region deleted in WAGR. Further evidence for a gene involved in Wilms tumour being located in 11p15 comes

from the association of 11p15 duplications or rearrangements in a subgroup of patients with the Beckwith–Wiedemann syndrome (BWS), a congenital syndrome of hyperplasia of kidneys and other internal organs, macroglossia, omphalocele, hemihypertrophy and predisposition to embryonic tumours such as hepatoblastoma, rhabdomyosarcoma and Wilms tumour. BWS may occur in families following an autosomal dominant pattern of inheritance with incomplete penetrance. The BWS predisposing gene is tightly linked to the loci for insulin and the Harvey-ras oncogene homologue *HRAS1* in band 11p15.5 (refs 10,11). The gene encoding insulin-like growth factor II (IGF2) can be considered a candidate because it maps very close to the insulin locus and is known to be expressed in fetal kidneys and in Wilms tumour. The genetic heterogeneity of predisposition to Wilms tumour is, however, not limited to genes in 11p13 and 11p15.5. Linkage studies of three families segregating for Wilms tumour, a dominant mutation with reduced penetrance, with no other abnormalities, have convincingly excluded linkage of the mutation or mutations present in these families to markers at either 11p site (refs 12,13).

How many genes are there in the human genome that, when mutated, will predispose to or cause Wilms tumour? Currently, we know of at least three. Do they all have to be changed for the neoplasm to develop? In the model of cumulative successive steps that has been proposed for adenocarcinoma of the colon, for small-cell carcinoma of the lung and other cancers of adult life, mutation or loss of alleles at loci on several different chromosomes is required for the stepwise development of the fully malignant phenotype. A model of parallel alternative pathways may, however, be more appropriate for embryonal cancers, such as Wilms tumour, that are either present at birth or detected early in life and where multiple genes, alternatively involved, could lead to a similar tumour. A less complex development of the retina compared with that of the kidney, requiring fewer essential steps, would explain why all mutations leading to retinoblastoma involve the same genetic locus. During renal development, on the other hand, genes at more than one locus are required, and if any one of them loses its function, incompletely differentiated cells would persist and could develop into a Wilms tumour in early childhood. A third model is that of hierarchical gene interaction. If the LK15 gene's function were to turn off a gene in 11p 15.5, let's say the IGF2 gene, in embryonic kidney cells at a certain stage of development, then loss of LK15 gene expression and mutation and allele loss at the IGF2 locus could have the same effect, but only events at one of the two loci

would be necessary. Under the last two models, each Wilms-tumour-predisposing gene present in each tumour would not have to lose its function.

How can these models be distinguished? The LK15 gene probe could be used to define a subgroup of sporadic or familial Wilms tumours in which this gene definitely does play a role. A possible correlation between the histological type of Wilms tumour and loss of LK15 gene expression, in conjunction with expression studies in normal embryonic kidneys, could provide clues to the stage of development at which this gene is important. It will be interesting to see whether homologues can be found in other organisms and whether they play a role in development. In particular, once the homologous gene has been cloned in the mouse, it can be mutated and a mouse model for LK15 deficiency (and, maybe, Wilms-tumour predisposition) can be created.

What is the normal function of Wilms-tumour genes? The observation that the non-random loss of genes or their expression is associated with certain solid tumours has led to the concept of tumour-suppressor genes, retinoblastoma being the prime example. The idea is further supported by experimental studies with somatic-cell hybrids between tumorigenic cell lines and normal cells in which retention of specific chromosomes from the non-tumorigenic parent, particularly chromosome 11, has been shown to suppress tumorigenicity, an effect that has been reproduced by introducing a normal chromosome 11 into a Wilms-tumour cell line¹⁴. The concept of tumour-suppressor genes suggests that nature has evolved to produce genes whose primary function is to suppress tumours, a view that is, in my

opinion, too narrow. It seems more likely that these genes are involved in essential differentiation steps. The inverse relationship between levels of differentiation and the growth rate and aggressiveness of tumours is well known in cancer biology. If the retinoblastoma or Wilms-tumour genes were to encode differentiation factors, their absence in certain cells of the embryonic organ would lead to the persistence of partially differentiated cells that may continue to divide. Islands of undifferentiated cells (blastema), present transiently in kidneys of normal newborns, have been shown to persist for a longer time in kidneys of children that are genetically predisposed to Wilms tumour. Putting the gene back into a retinoblastoma or Wilms-tumour cell line may simply help the cells to complete a necessary differentiation step that simultaneously turns off its malignant growth potential. □

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ENZYMOLGY

More of the catalytic triad

David Blow

It is just over 20 years since the discovery of the charge-relay system (or 'catalytic triad' as it is often described) at the active site of the serine proteinases. Now, two independent structure determinations of lipases^{1,2} are reported on pages 767 and 771 of this issue; they show the same constellation of aspartic acid . . . histidine . . . serine activating a catalytic serine that is found in the serine proteinases.

At the active site of every serine proteinase, there is a serine which is within hydrogen-bonding distance of a ring nitrogen of histidine — invariably N¹ in chemical nomenclature (N² or N⁰¹) is hydrogen-bonded to the carboxylate group of an aspartate which, exceptionally for acid groups in proteins, is buried. This system was discovered, at

almost the same time, in the structures of α -chymotrypsin and of subtilisin BPN' (refs 3,4), the first two serine proteinases whose three-dimensional structures were known.

Over the years there has been considerable discussion about the precise significance of the charge-relay system and the distribution of charge within it. Kossiakoff and Spencer⁵ showed by neutron diffraction that the histidine was fully protonated at N⁰¹, and Craik *et al.*⁶ engineered an Asp→Asn mutant in rat trypsin. This mutant showed that the polarization of the histidine by the buried aspartate enhanced the reactivity of the serine by about three orders of magnitude. Warshel⁷ has used the techniques of computational chemistry to estimate the effect of the charged carboxylate group and the polarized histidine

on the reactivity of the serine side-chain surrounded by water.

If the histidine rings of subtilisin and an enzyme of the trypsin family are superimposed, the hydrogen-bonded oxygens of the aspartate and serine are also superimposed. This is a geometrical consequence of strong hydrogen bonding, and the similarity ends there. The other side-chain atoms, and the directions of the main chains carrying the triad, are very different in orientation. Because there is no similarity whatever in their overall structures, subtilisin and the trypsin family have become the most quoted example of 'convergent evolution' at the molecular level.

The lipases now form part of this example of convergence. Their overall structure appears to be quite unrelated to either of the serine proteinase families. Although strong hydrogen bonding creates the same geometry around the histidine, the extent of structural similarity elsewhere within the triad is not yet completely clear: Brady *et al.*¹ stress that the side chains of His and Asp are in almost the same conformation as in *Streptomyces griseus* trypsin, whereas Winkler *et al.*² show that all the side chains are in different conformations from those in bovine trypsin. But in all charge-relay systems the histidine is the same way round, with N^{δ1} facing the carboxylate, and in all of them the carboxylate is aspartate not glutamate. Why is this?

Assisted by Janet Thornton, I have searched the Protein Data Bank to see whether any other Asp . . . His . . . Ser constellations are lurking there. Two candidates emerged. In taka-amylase⁸ there is a constellation in which the histidine is reversed, and the hydrogen bond to serine must be very weak (length over 3.4 Å). There is no suggestion that this histidine (His 108) has any catalytic function. In the immunoglobulin Kol⁹, there is another Asp . . . His . . . Ser, with the histidine (His 225, heavy chain) the 'right' way round and shorter hydrogen-bond lengths; but the geometry is worse, because both Asp and Ser are far from the plane of the histidine. This discovery prompts the thought that immunoglobulins, with their rich mechanism for variability, might frequently produce catalytically active con-

formations by chance. But even if such a catalyst were wonderfully advantageous for the cell line that produced it, there would be no mechanism for the host's progeny to reproduce it.

An important feature of the lipases is that the supposedly 'active' serine is completely buried by a loop or 'lid' which must be moved to give access to a substrate. Winkler *et al.*² have formed a butylboronate adduct of the serine in crystals, but only at low occupancy. They suggest that a major conformational change, involving at least three segments of chain, would be needed for a triglyceride to reach the active serine.

Many amino-acid sequences exist for lipases: there are different lipases in

hepatic, pancreatic and endothelial cells of mammals, and there is evidence that they arise from divergence of splicing sites within the introns and exons of the gene¹⁰. The catalytic serine of lipases invariably occurs in the sequence Gly-X-Ser-X-Gly, reminding one instantly of the trypsin family's characteristic Gly-Asp-Ser-Gly-Gly. Subtilisin, on the other hand, has Gly-X-Ser-X-Ala. Because the main-chain conformations at the serine are so different, can the similarity of sequences between families be anything more than a coincidence? Whatever the answer, we shall hear more of Asp . . . His . . . Ser. □

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HIGH-TEMPERATURE SUPERCONDUCTORS

Flux lines traced out

David Caplin

SUPERCONDUCTORS that retain their superconductivity to high magnetic fields do so by allowing the field to permeate the material, and so avoid incurring the energy penalty of total field exclusion. This is the mixed state of type 2 superconductivity. In both conventional low-temperature superconductors and the new oxide high-temperature superconductors, it is well established that the field penetrates as quantized flux lines, or current vortices. The question of how the flux lines arrange themselves, and how they move, is a central one, and affects the prospects for the eventual application of the new materials. Now, the existence of an ordered flux-line lattice in a single crystal of YBa₂Cu₃O₇ has been confirmed with a neutron diffraction experiment, as reported by Forgan *et al.* on page 735 of this issue¹. Although the flux-line lattice had been observed previously in YBa₂Cu₃O₇ by other techniques, neutron diffraction offers the prospect of much more detailed information about the ways in which the lattice can be disrupted, which is important in determining the critical current of these materials.

The main obstacle standing in the way of technological applications of the new superconductors, such as YBa₂Cu₃O₇, which has an attractively high transition temperature, T_c , of 92 K, is their generally poor critical-current performance, particularly in magnetic fields². This problem arises because, in the mixed state of the new materials, the flux lines seem to be far more mobile than in the conventional superconducting alloys based on niobium that have T_c s below about 20 K. Flux lines move in response to the Lorentz force between them and the current, and so, by Faraday's law of induction, induce a voltage. Although the material remains superconducting, in the sense that its elec-

trons are ordered into a state that differs from the normal state, it then loses its all-important property of zero-resistance.

There are several experiments³⁻⁵ that suggest that a modification is needed to the usual magnetic-field-temperature phase diagram of a superconductor (see Fig. 1), with a demarcation line between a dissipative, mobile-flux region, and one where the flux is immobile and the material carries a current without loss. The interpretation of this phase diagram is hotly debated, for it is unclear whether the mobile-flux regime is merely much more extensive than in conventional super-

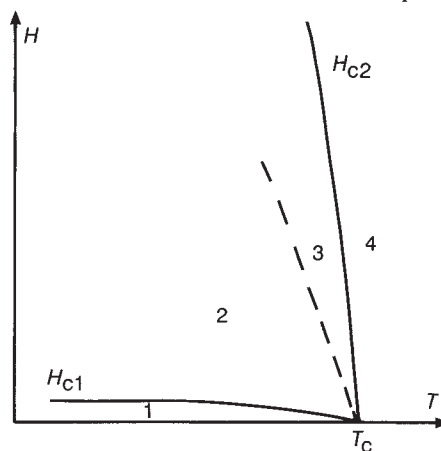


FIG. 1 The magnetic-field-temperature phase diagram of a type 2 superconductor. Region 1 is the Meissner state of total magnetic flux exclusion. In regions 2 and 3, the mixed state, the flux penetrates but is immobile (region 2) or mobile (region 3); consequently there is zero resistance in region 2, but not in region 3. Region 4 is the normal state. An important difference between the new high-temperature oxide superconductors and low-temperature type 2 materials, such as those based on niobium that are used for superconducting magnets, is that region 3 is extensive in the former, but very small in the latter.

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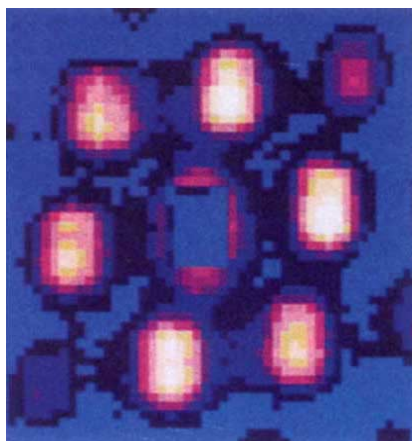


FIG. 2 The diffraction pattern of neutrons scattered by the hexagonal flux lattice of niobium. The niobium was in a field of 2 kilogauss and at a temperature of 2 K.

conductors (in which the effects of moving flux are apparent only in a very narrow range of field and temperature), or if a new description is needed.

Because of their magnetic interactions, the flux lines certainly do tend to form an ordered, usually hexagonal, array. This flux-line lattice (FLL) in $\text{YBa}_2\text{Cu}_3\text{O}_7$ crystals has been observed by several groups^{6,7}, using a magnetic decoration technique, in which fine ferromagnetic particles are evaporated onto the crystal; these congregate at the entrance and exit points of the flux lines from the surface. Does the new demarcation line on the phase diagram represent the melting curve of the FLL, or is there perhaps an additional flux-line 'glass' phase, with the flux lines frozen but disordered⁸? Or, do extensions of the ideas of flux-line pinning at defects and pinning of the FLL, drawn from work on conventional superconductors, provide a sufficient description? The latter view⁹ regards the distinction between mobile and immobile flux as being primarily one of timescale, in which the average rate at which flux lines jump from one pinning site to another depends exponentially on the temperature and other parameters. The situation is complicated further in the new materials by their severe anisotropy, which causes the energy of flux lines and the interactions between them to depend strongly on their orientation with respect to the crystal lattice^{10,11}.

The neutron-diffraction experiment of Forgan and collaborators is important because it can, in principle, help to answer these questions. The magnetic decoration technique has produced beautiful evidence of the FLL, but is limited in several respects. It takes a 'snapshot' of the flux-line arrangement at the temperature of decoration, and so cannot follow any temperature dependence of the flux-line order. It has rather limited spatial resolution, and so cannot 'see' flux lines at even moderately high fields, when they crowd

together. Finally, there is always the possibility that the surface pattern is not representative of the bulk behaviour.

Neutrons, because of their magnetic moment, are sensitive to the spatial variation of the field in the mixed state, and this technique for the observation of the FLL is well established in conventional superconductors¹² (Fig. 2). However, the experiment is a difficult one in $\text{YBa}_2\text{Cu}_3\text{O}_7$; because the field associated with a flux line varies over a distance of the order of the field penetration depth λ , which is several times larger in $\text{YBa}_2\text{Cu}_3\text{O}_7$ than in conventional superconductors, the magnetic contrast of the FLL is reduced greatly. Also, a single-crystal sample is essential if well defined diffraction peaks are to be observed, and crystals of the new materials are notoriously difficult to grow to a size sufficient for neutron-diffraction studies.

The results of Forgan *et al.* demonstrate that, at low temperatures, an ordered FLL has formed in their sample; but, because their crystal was heavily twinned (that is, it had regions in which the crystallographic *a*- and *b*-axes are interchanged), their diffraction pattern displays the 4-fold rotational symmetry of the twinned crystallographic lattice, rather than the (nearly) hexagonal symmetry of the FLL.

GLOBAL WARMING

Beware greenhouse confusion

T. P. Barnett

WITH public debate growing about the greenhouse effect and the possibility that carbon dioxide is causing global warming, many will be interested to read, on page 709 of this issue¹, the sophisticated analysis by Kuo *et al.* of the trends in global mean temperature and concentrations of CO_2 at Mauna Loa in Hawaii. The authors find significant trends in each data set and strong correlations between them at time-scales of a year and more. But despite caveats in the text discussing the analysis, readers might be left with the impression that the results support the idea that greenhouse-gas-induced global warming has been observed with high statistical confidence. This is by no means the case.

The potential threat posed by greenhouse warming is unlike any other faced by mankind, for it could affect every aspect of our civilization. If we are to decide how to confront it, we must first assess the magnitude of the threat. In other words, are the climate models even approximately correct in their greenhouse predictions? If so, can the predicted climate changes be seen in the real world already? The latter is the important question that Kuo *et al.* have tried to tackle.

The fact that both the temperature and the CO_2 time series they have studied

that has been seen in the flux decoration experiments^{6,7}. The temperature dependence of the diffracted intensity appears to deviate from that which would be expected if the FLL survived all the way up to T_c , which is perhaps indicative of flux lattice melting.

Now that neutron diffraction of the FLL in $\text{YBa}_2\text{Cu}_3\text{O}_7$ has been shown to be possible, we can anticipate that when larger single crystals of high quality become available, the current arguments about the way flux lines behave in the new superconductors will be resolved. □

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show a positive trend does not imply that greenhouse warming has been observed: the population trend of San Diego is also positive over the same period (1958 to the present), but no one is suggesting that it is responsible for the apparent increase in global temperature. Furthermore, the 'global' temperature data² used in the analysis are entirely from land stations and so ignore temperature changes over the oceans — roughly 72 per cent of the Earth's surface.

A simple index such as the mean global temperature is affected by many processes³ and so is not an adequate detector for greenhouse effects. Contamination can arise from time-dependent biases in the measurement technique (which may introduce spurious trends), changes in cloud cover, El Niño events, volcanoes, atmospheric turbidity and so on. Each item could raise or lower the estimated global temperature. But if we see a change in the temperature, how do we know which process to attribute it to? The single number is insufficient to allow us to decide.

So it is virtually impossible reliably to attribute a cause to the observed changes. Perhaps the only way to detect a greenhouse signal convincingly is through a simultaneous four-dimensional analysis of

multiple signals predicted *a priori* by an adequate climate model⁴. Virtually all such models show the greenhouse signal to have substantial spatial structure. For example, the polar regions are predicted to be warmed more than the tropics; and land masses are warmed more than the surrounding oceans. These features could be the unmistakeable fingerprints of greenhouse warming, but are lost by looking at global averages. They may also be unique to the greenhouse effect, allowing other warming mechanisms to be ruled out. This critical problem of attribution underlies all attempts to say a greenhouse warming has been observed.

Kuo *et al.* draw out one feature of their data partially linking the global CO₂ budget to large-scale changes in temperature. As they note, changing temperatures will lead to changes in the amount of CO₂ in the atmosphere, owing to outgassing or dissolution of CO₂ in the oceans and variations in biological activity. Because such temperature changes occur over a broad range of characteristic times, one would expect to find a similar broad spectrum in the CO₂ response. Indeed, the authors find such a spectrum, so that the CO₂ data are correlated, positively, with the temperature changes—but they lag behind by 5 months, suggesting that the causality is temperature-to-CO₂. Those seeking evidence of greenhouse warming would expect the opposite. Thus, much of the broadband coherence between the two variables may be due to temperature changes arising from natural climate variability and has nothing to do with greenhouse effects.

The powerful statistical methods Kuo *et al.* apply to the painfully short, heavily flawed instrumental climate record are to be welcomed. Studies of this kind are essential if we are to assess the potentially awesome consequences of the greenhouse threat. Unfortunately, it is easy to become mesmerized by technique and to overlook the physical problems that may bias the crude data towards one disposition or another. More risky still is the rush to use the new methods to reach a trendy conclusion, one that is not solidly backed by physical reasoning. The authors have tried to avoid such pitfalls by expressing appropriate caveats. As long as these are picked out, no one should gain the impression that the paper shows the first evidence of CO₂-induced warming. It doesn't. □

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Sometimes an editor makes sense

Charles Weissmann, Roberto Cattaneo and Martin A. Billeter

PRINCIPLES of molecular biology once seemed to be simple and straightforward. The co-linearity principle implied that if the nucleotide sequence of a gene were known, the sequence of the cognate protein could be predicted. The discovery of split genes and the necessity of either rearranging segments at the DNA level or splicing coding domains of the pre-messenger RNA showed that this was not always so. Still, the conversion of a primary transcript to the mature, translatable mRNA could be ascribed to sequence information contained in the pre-mRNA and therefore in the cognate gene. The discovery of massive discrepancies between the nucleotide sequences of certain trypanosomal genes and the proteins they were believed to encode^{1–3} has added a further level of complexity. Extensive sequence changes or 'editing' are necessary to generate sensible mRNA from the primary transcripts, which lack features essential for correct translation, such as an initiation signal or an appropriate reading frame. Who is the 'editor' and where does the information come from that allows the conversion of nonsense to sense? Writing in *Cell*, Simpson and colleagues⁴ now propose that the editorial information is not contained in the primary transcript or in the gene encoding it, but is scattered in pieces over the genome, and that the editor is a nucleoprotein consisting of nuclease(s), nucleotidyl transferase, RNA ligase and a short instructive RNA molecule.

What led to this proposal? The story begins in 1986 with the startling finding by Benne *et al.*¹ that although *Trypanosoma brucei* contains an mRNA encoding mito-

chondrial cytochrome oxidase subunit II (COII) in a perfectly conventional manner, the COII gene itself, which is located on the mitochondrial maxicircle DNA, lacks four (closely spaced but non-contiguous) uridylyte residues within the coding sequence; this not only modifies the deduced protein sequence locally, but also alters the downstream reading frame. Trypanosomatid protozoa possess a single, complex mitochondrion which is located at the base of the single flagellum and contains two kinds of circular DNAs: 20–50 copies of a single type of maxicircle and about 10,000 copies of more or less heterogeneous minicircles, interlocked into a network. The information content of the minicircles is unknown; the maxicircle carries some 'normal' genes, the 'defective' COII gene and, as shown later, several other defective genes. Benne *et al.* convincingly demonstrated that there is no 'correct' COII gene hidden in the trypanosome and concluded that the primary COII transcript is edited by co- or post-transcriptional insertion of four U residues. RNA editing in trypanosome mitochondria based on the insertion (and, as shown later, excision) of U residues was soon extended by the additional examples of the cytochrome oxidase subunit III (COIII), apocytochrome *b* (CYb), MURF-2 and MURF-3 transcripts³.

The most extreme case of uridylyte-based editing found so far is that of the COIII gene. It had been noted earlier that maxicircle DNA of *T. brucei* contains all the same genes that are contained in the maxicircle DNA of *Leishmania tarentolae*, except for the COIII, MURF-3 and MURF-4 genes, which are replaced in the

Different types of editing

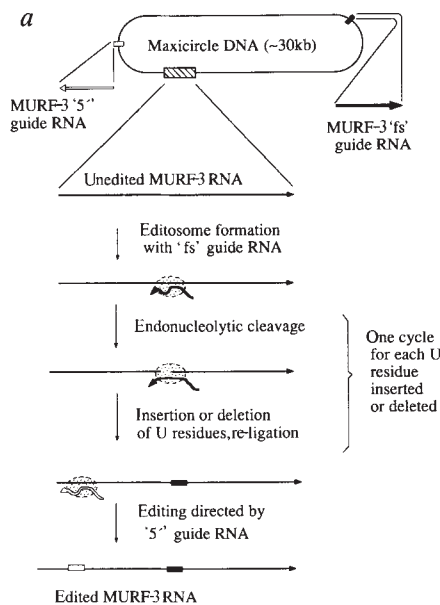
Type of editing	Mechanism	Source of editorial information	Occurrence
Insertion, deletion of one or more U residues ^{1–4}	Cleavage, U addition or deletion, ligation	Extragenic (guide RNAs)	Mitochondrial mRNAs of trypanosomatids
C replaced by U (or U-like base) ^{10–14}	Deamination (?)	Intragenic (secondary or tertiary structure ?)	Apolipoprotein-B mRNA in mammalian cells Mitochondrial mRNAs of plants
Insertion of one or more G's ^{15–17}	Co-transcriptional polymerase stuttering	Intragenic	Paramyxovirus mRNA
3'-terminal A addition ^{19–20}	Polyadenylation	Intragenic (RNA terminus)	Mitochondrial mRNAs of mammals

Messenger RNA editing is defined as a process leading to pre-determined modifications of the coding region of a primary gene transcript. By this definition, splicing processes are special forms of editing but are not dealt with in this table.

maxicircle of *T. brucei* by shorter, G+C-rich regions⁵. Two years ago, Feagin *et al.*² found mRNA encoding COIII in *T. brucei*; the sequence of this mRNA could be aligned with the sequence of the genomic G+C-rich region that replaced the COIII gene in other trypanosomes if 398 T residues were added at 158 sites and 19 T residues were deleted at nine sites in what was later called a 'cryptogene'. The conclusion that the cryptogene transcript is edited by appropriate addition and deletion of U residues is supported by the finding of not only 'unedited' COIII RNAs corresponding to the genomic sequence, but also of what seem to be partially edited RNAs. It is interesting that such putative intermediates were sometimes found to contain an excessive number of inserted U residues, as though the editor had done too much of a good job. The distribution of edited stretches indicated that editing commences at the 3' terminus and moves upstream. Feagin *et al.* suggested that the editing mechanism consists of the identification of the improper sequence, cleavage, either addition or trimming of U residues to give the proper number of U residues, and re-ligation. But the source of information required to instruct the editing mechanism remained a mystery because no 'correct' DNA sequence could be found, nor was there evidence for a 'correct' RNA minus strand that could serve as template for editing or for replication. Desperate ideas were invoked, such as signals on the unedited RNA capable of instructing the 'editor', or information transfer from the edited plus strands mediated by mechanisms not involving base pairing.

What have Simpson and his colleagues³ achieved? They reasoned that if no complete 'correct' gene copy or complementary RNA existed, maybe at least short segments of DNA corresponding to the edited portions of primary transcripts could be found. Indeed, a computer search of the 30-kilobase (kb) *L. tarentolae* maxicircle sequence revealed seven short sequences that could potentially yield RNA fully complementary to the edited regions of the CYb, MURF-2, MURF-3 and COII primary transcripts, provided that G-U base pairing was allowed for. Using plus-strand probes corresponding to these seven regions, short RNAs of less than 80 nucleotides were identified and characterized for each of these edited regions. Because of their presumed function, these RNAs were called guide RNAs, or gRNAs for short.

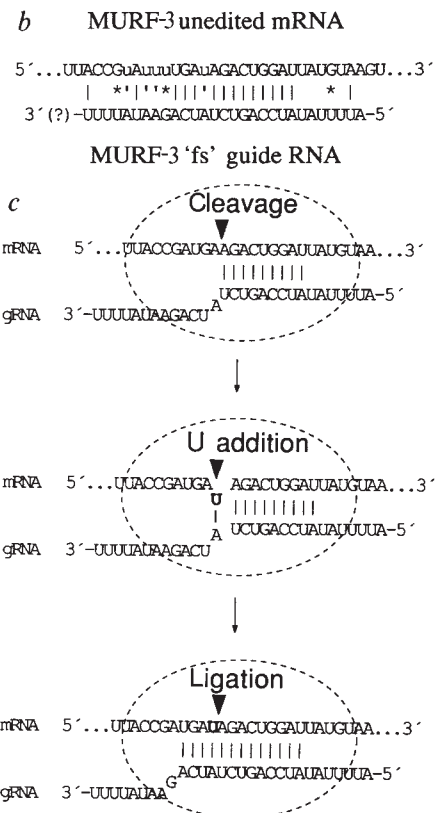
The figure (a, top) shows the proposed editing mechanism, using as an example the MURF-3 transcript (edited MURF-3 transcript encodes a polypeptide homologous to a subunit of NADH dehydrogenase). The mitochondrial maxicircle DNA of *L. tarentolae* contains the MURF-3 cryptogene (hatched box) whose sequence



Model for RNA editing in trypanosomatid mitochondria, as proposed by Blum *et al.*⁴. a (top), Location of the sites corresponding to MURF-3 mRNA and gRNAs on the maxicircle DNA of *L. tarentolae*. The arrows indicate 5' to 3' polarity. a (below), Steps in the editing process. b, Sequences of a segment of the unedited MURF-3 mRNA and of the entire gRNA. Residues added by editing are shown by a lower-case u in the mRNA. Standard complementarity is indicated by long vertical lines, U-G pairs present only after editing by short vertical lines. c, Detail of one cycle of RNA editing.

differs from that of the mature mRNA in two regions (designated '5'' and 'fs'); this requires the introduction into the primary transcript of 20 and 5 U residues, respectively. The DNA segments (white and black boxes) corresponding to the two MURF-3 gRNAs are located about 3.5 kb and 14 kb away, respectively, and are transcribed in opposite orientations. In each MURF-3 gRNA there is a segment complementary to a stretch (in this case of nine nucleotides) 3' to the region to be edited (b in the figure). The MURF-3 'fs' gRNA and the mRNA are aligned in the context of an 'editosome' (stippled oval in the figure, a,c), a conjectural ribonucleoprotein particle containing the gRNA and various enzymes. An endonuclease cleaves the unedited RNA 3' of the first base not paired with the gRNA, and a terminal uridylyl transferase inserts a U nucleotide into the mRNA opposite an A or a G in the gRNA (c in the figure). If the mismatch in the mRNA is due to a U, this residue is excised by a conjectural exonuclease, or perhaps by the uridylyl transferase itself. After joining by an RNA ligase, a new cleavage and editing cycle is initiated at the next mismatch.

This editing model is based on the identification of the gRNAs with regions complementary to the edited mRNA segments (allowing for G-U base pairs), a terminal uridylyl transferase activity in the mitochondria of *L. tarentolae*⁶, and an RNase P-like ribonuclease believed



to cleave 3' of the first mismatched nucleotide.

Is this all there is to the synthesis of edited RNAs in trypanosome mitochondria? Perhaps not. Guide RNAs for the editing of the COIII transcripts have not yet been identified, but they could be present on minicircular or even nuclear DNA and difficult to find experimentally because of the complexity of the DNA and the necessity of allowing for G-U base pairing. Alternatively, a different editing mechanism could be invoked. Volloch *et al.*⁷ make the startling proposal that unedited COIII RNA of *T. brucei* is not the immediate precursor of the bulk of its edited counterpart, but that the latter is synthesized from an edited template (such as a full-length, edited minus-strand RNA) in an actinomycin-resistant process. This claim is based on the finding that actinomycin strongly decreases the level of ³²P-labelled unedited RNA, but not ³²P-labelled edited RNA, as measured within a three-hour period of labelling with ³²P. Because the half-lives of the two RNA species are not affected by the drug, the observations are ascribed to a selective inhibition of DNA-directed synthesis of unedited RNA. The authors argue against the possibility that, in the presence of actinomycin, a pre-formed pool of unedited RNA is edited (and ³²P-labelled by pU residues) by showing that the four nucleotides in edited RNA have the same specific radioactivity, which should not be

the case if labelling were due only to the insertion of [^{32}P]pU residues into an unlabelled precursor; we did not find this experiment technically fully convincing, however. If the claims of Volloch *et al.* are borne out, they could be indicative of a different mechanism, but they could also be reconciled with the model of Blum *et al.* by assuming that editing of the primary transcript occurs as proposed by the latter, but that subsequently amplification of the edited (and perhaps intermediate forms) occurs by RNA-dependent RNA synthesis. Volloch and his colleagues earlier proposed that β -globin mRNA in reticulocytes is amplified by an RNA-dependent replicase^{8,9}, a suggestion that remains to be confirmed.

Several other types of editing are listed in the table on page 697; in all these cases the editing process probably does not require extragenic sequence information. A single C to U conversion in the apolipoprotein-B mRNA of intestinal (but not of liver) cells, generates a termination codon and thereby a truncated version of the protein^{10,11}. This base change may be caused by a deaminase recognizing some structural feature of the mRNA, much as in transfer RNA modification in both eukaryotes and prokaryotes. A similar mechanism may underlie the frequent editorial C to U changes in plant mitochondrial mRNAs, which result in altered meaning of individual codons^{12–14}. 'Stuttering' of the RNA-dependent RNA polymerase at a particular intergenic region of paramyxovirus genomic minus-strand RNA introduces non-templated G residues and thereby generates mRNAs encoding one or more additional proteins^{15–17}.

What is the evolutionary origin of this strange, template-directed editing process in trypanosomatid mitochondria? It is clear that the process must have originated earlier than at least 200–300 million

years ago, because trypanosomatid species (*Crithidia fasciculata* on the one hand, and *T. brucei* and *L. tarentolae* on the other) diverging at about the same time¹⁸ all possess the U-based mechanism³. The COII 'gene defects' are also at least that old, because they are identical in the trypanosomatid species mentioned above. But in the case of COIII gene of *T. brucei*, most of the many deletions and additions must have arisen more recently than 100–150 million years ago, because they are not found in *L. tarentolae*, which diverged from *T. brucei* at about that time. These mutations are compatible with survival of the organism only if an editing mechanism that could deal with the defective COIII transcripts already existed in the mitochondria. If the mechanism of Blum *et al.* were responsible for this editing, COIII-specific guide RNAs would have to pre-date the mutations; this suggests that the guide RNAs survived from an earlier evolutionary period,

COSMIC STRUCTURE

Looking backwards past zero

Marc Davis

LOOKING for patterns in the distribution of matter in the Universe is an old game. After the Milky Way was recognized as a massive collection of stars — our Galaxy — clusters of galaxies became identified. And then superclusters of clusters. As they describe on page 726 of this issue¹, not only have Broadhurst *et al.* found structure at the largest scale they have studied, but they find periodic oscillations of density. If the galaxy distribution is truly periodic, it is safe to say we understand less than zero about the early Universe.

The past decade has been an active one for cosmic cartographers, as the advent of efficient linear detectors has made astrophysical spectroscopy and the determination of galactic redshifts a routine and relatively simple observational task. The redshift z of a galaxy is, to first order, proportional to distance and is defined simply by the displacement of its spectral-line wavelengths (λ), $z = \Delta\lambda/\lambda$. A large amount of time on moderate and large aperture telescopes has yielded redshifts of more than 10,000 galaxies, with several thousand more accumulating each year. The Universe on small scales is extremely clumpy, and the redshift surveys provide invaluable three-dimensional details of the clustering of galaxies that cannot be inferred merely from the two-dimensional distribution of galaxies on the sky. Geller and Huchra, in a recent review of this work², argue that the largest structures observed always seem to be close to the limit possible in a given survey, and that the size of the limiting

where their presence may have been essential for the assembly of the primaevial COIII mRNA. Alternatively, some other novel editing mechanism which does not depend on COIII-specific guide RNAs would have to be invoked.

Trypanosomes differ from other protozoa by the nature of their single, flagellum-associated mitochondrion, which now proves to contain this unique editing mechanism. The mitochondrion and its flagellum may have originated from an ancient, free-living cell, and thus the template-directed editing process may be derived from a primaevial mechanism that originally served to generate maximal sequence diversity from RNA of limited length. Self-splicing and trans-splicing may be relics of other processes serving the same purpose. □

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structures shows no evidence of convergence on large scales.

Most redshift surveys to date have been either shallow ($z < 0.03$) three-dimensional surveys of a few thousand galaxies covering a large solid angle, or somewhat deeper ($z \geq 0.05$) two-dimensional surveys covering a narrow strip of the sky. The new report by Broadhurst *et al.* is on a deep one-dimensional redshift survey extending to redshift $z = 0.5$ in an area of less than one square degree. The authors have surveyed several hundred faint galaxies in small patches at the North and South Galactic poles and here combine their results for the first time. Not only does the redshift distribution reveal the expected strong clustering, but a good fraction of the galaxies seem to be regularly arrayed in compact clumps with a uniform distance spacing of 120 megaparsecs (Mpc). This remarkable periodic pattern can be traced across ten consecutive peaks and appears phase coherent in both directions of the sky.

The question of large-scale structure has been a very active and vital field of astrophysics for the past decade, partly because the improved technology made large redshift surveys possible just as theoretical ideas related to cosmic inflation and early Universe physics were being developed³. The observed large-scale structure is largely an initial-value problem, because matter has not moved sufficiently far to erase the initial pattern of large-scale perturbations after the Big Bang. One of the attractions of the inflationary cosmology is that the initial fluctu-

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ations which later became galaxies and clusters of galaxies can be explained as residual quantum noise generated during the inflationary period of the very early Universe; previously cosmologists had no mechanism to provide a dynamical explanation for the initial fluctuation spectra and simply had to posit them as initial data laid down by the 'hand of God'. Although there exists no detailed theory of inflation, the overall concept is extremely attractive because it not only suggests a mechanism for fluctuation generation, but it simultaneously provides dynamical explanations of the large-scale homogeneity and 'flatness' of the Universe (the fact that the observed density is close to the critical value for a gravitationally closed Universe).

The generic fluctuations expected from this theory are characterized as gaussian random noise — and most definitely are not periodic. The simplest model of clustering consistent with inflation is the cold-dark-matter model, which has had considerable success at describing clustering on scales less than 10 Mpc, but the predicted fluctuations on a large scale are perhaps insufficient to explain recent observations¹. Alternative models for large-scale structure based on initial 'isocurvature' fluctuations, explosive hydrodynamics, and bizarre topological defects such as 'cosmic strings', 'domain walls', or even 'textures' have also been proposed to explain large scale structure². Some of these models can produce non-gaussian initial fluctuations, but none of these ideas leads to periodic structure. Therefore validation of the claim of Broadhurst *et al.* is a matter of some considerable importance.

Before overturning all our preconceptions, we should consider the significance of the finding. Right away we encounter a fundamental roadblock admitted by the authors: the statistics are all *a posteriori*, as nobody expected to see periodic redshift distributions. To me, the situation is reminiscent of the Nemesis affair, where a variety of models were invented to explain an apparent 26-million-year periodicity³ in the rate of species extinction of the Earth's fossil record. In that case, like here, a power-spectral analysis showed a very significant peak. Corroborating evidence for periodicity in the ages of craters seemed to confirm the extinction data⁴. Since the heat of that discussion dissipated, most interested bystanders have taken the view that the evidence for individual comet showers is excellent, but that the case for periodicity is not compelling.

In the current case, corroborating evidence takes the form of shallower redshift-survey data over a somewhat larger field of view. The shallower data show an excess of galaxies at the positions of the nearest of Broadhurst and colleagues' peaks, but many other clusters are present

as well and the periodicity is not conspicuous. It is unclear how to interpret a periodic distance distribution. Does it imply that galaxies are distributed on uniformly spaced cell walls, or does it imply that the topology of space is multiply connected, as once suggested by Fang Li Zhi to explain an apparent periodicity of quasar redshifts⁵? If the galaxy distribution is cellular, then the wavelength of periodicity will depend on the angle of the probe with respect to the normal axes of the cells. On the other hand, if the present result is a statistical fluke, then future surveys in different directions will continue to find strong clustering, but no periodicity. At that point we shall have another demonstration of the fact that unusual events happen *a posteriori* all the time and that a significance level of around 3×10^{-4} should not necessarily be taken as compelling.

The original motivation of the pencil-beam redshift surveys was to study the evolution of field galaxies to reasonable distance; at the time the surveys were begun most observers expected the galaxy distribution along a typical beam would be only modestly clumpy. With hindsight, however, it is not surprising that the distribution is so dramatically irregular. The clustering of galaxies is known to be of sufficiently large amplitude for most of space to be empty. Two- and three-dimensional redshift surveys show that most galaxies are arranged on sheets and filaments that surround and interpenetrate voids of typically 30 Mpc across, giving a sponge-like topology. A narrow-beam survey may traverse several voids before encountering clumps of galaxies, so that voids apparently on a scale of 100 Mpc in such surveys can be commonplace. As a consequence of these excessive fluctuations, estimates of the correlation amplitude derived from narrow beam surveys are less stable than those inferred from two and three dimensional surveys. Narrow beam surveys like the kind reported by Broadhurst *et al.* are most useful for constraining the evolution of galaxy clustering, as they are the only galaxy surveys that probe to sufficient distance and lookback time for evolution to be evident. □

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Virtuous vice

THE puritan view of life is that nice things are bad for you, whereas "If it hurts it's doing you good". Daedalus is puzzled how such a self-defeating state of affairs could have evolved; but it seems to apply depressingly well to the business of nutrition. The healthiest foods — the high-fibre, high-tenacity, low-calorie, low-taste vegetarian ideals — are nearly all desperately boring. By contrast, the rich, meaty, fatty, sugary confections we all naturally enjoy are the subject of endless medical denunciation. And the addictive drugs like nicotine and alcohol and cocaine, pleasurable enough to be the centre of a trillion-dollar industry, kill their devotees in millions.

There has to be a middle way. That ancient addictive drug alcohol used to be tamed simply by high dilution. Traditional British 'small beer' had so little alcohol in it that even as a staple drink it only slightly tempered its users' sobriety. The same strategy protects the coca-chewing peasants of South America; only in extracted, concentrated form is cocaine really dangerous.

So on balance, says Daedalus, human health would be greatly improved if the healthy boring diet recommended by modern nutritionists were actually made attractive by small additions of addictive drugs. At first he contemplated crossing tobacco with spinach or coffee bushes with lentils, to create habit forming, if not actually addictive, health vegetables. More fundamentally, the genes for the various addictive alkaloids could be extracted from the drug plants, and inserted by genetic engineering into the healthiest of the staple food crops.

Caffeine and cannabinal seem the alkaloids of choice. Coffee is pretty harmless, and marijuana probably damages its users more by smoke inhalation than alkaloid poisoning. So DREADCO dieticians are adding trace quantities of caffeine and cannabinal to various repulsively healthy diets, to discover how easily obese volunteers can stick to such diets; and whether they notice anything odd.

Daedalus reckons his volunteers will notice nothing, any more than tea or coffee drinkers recognize why these curious-tasting liquids should be so acceptable. They will merely congratulate themselves on their new-found will power. If so, Daedalus will launch his addictive health food on the market, or at least on the food-additive and regulatory medical authorities. With their strong belief in obedience, will power and self restraint, at least for their customers, the medics will put up a stiff fight. But the unprecedented prospect of a healthy diet you can actually live with should appeal so strongly to those same customers that Daedalus is hopeful of winning.

David Jones

Balancing background noise

SIR—I had not known, until Huxley's comment¹ on my article², of Bárány's suggestion that the auditory ossicles could balance out background noise, although I naturally knew of his important work on bone conduction.

Because of the past history of the Mammalia, three auditory ossicles form the middle ear transformer. All other tetrapods have only one. The mammals evolved from mammal-like reptiles, which had the tympanic membrane in the lower jaw, supported by the angular bone^{3,4}. Sound was conducted from the tympanic membrane through the jaw articulation, formed by the articular and quadrate, to the stapes, which itself articulated with the quadrate.

Mammalia, by definition, have a secondary jaw articulation, between squamosal and dentary, which replaces the primary jaw articulation between quadrate and articular. With the new articulation, the ear drum moved from the lower jaw to the skull, the angular became the tympanic and the articular and quadrate the malleus and incus. So the mammals came into being with the chain of three bones in the middle ear available both to increase the transformer ratio and to function as suggested by Bárány.

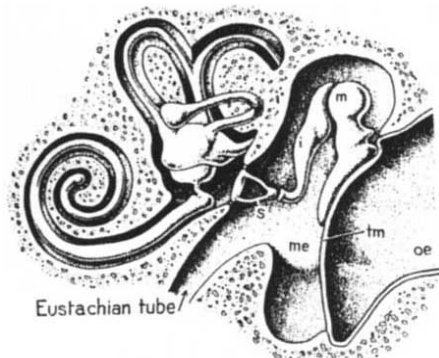
The problem of background noise was much more acute in the mammal-like reptiles than in their successors, the mammals. Because the articular bone moved during mastication, it would have been essential not only to balance out unwanted noise, but to prevent damage to the organ of Corti from overstimulation by the inevitable gross movements of the stapes.

I have always been aware of the problem of unwanted noise in these reptiles, but had thought that it could be solved by some form of nerve block, rather as unwanted noise can easily be filtered out in an electronic circuit. I did not consider the question of damage to the inner ear, where some mechanism such as that suggested by Bárány could well have been important.

In present-day lizards, the tympanic membrane is held in a frame formed by the quadrate bone supplemented posteriorly by soft tissues, and in particular by a strong band of connective tissue attaching ventrally to the retroarticular process of the lower jaw. Mastication, when both the quadrate and the articular move, occasions two problems: suppressing the consequent background noise, and preventing damage to the inner ear.

In lizards, sound is conducted from the tympanic membrane to the inner ear by the stapes and a cartilagenous extra-stapes; sometimes there is a movable joint between them. Wever⁵ discusses this condition in the iguanid *Crotaphytus*, where

the lever action between stapes and extra-stapes gives a transformer ratio of two and a total transformer ratio in the middle ear of 26.6 — well up into the mammalian range (man has a transformer ratio of only 18.3). Wever does not consider the problem of background noise or damage, but I have little doubt that the mechanism postulated by Bárány could be effective. I do not know whether the mechanism would suffice in *Chameleon*, where the



Diagrammatical section of the middle ear in a mammal, showing the otic region.

stapes rocks on the rim of the fenestra vestibuli, giving the high transformer ratio of 5.6 (ref. 6).

Gekko, with the highest reptilian transformer ratio⁵ of 39.9 and a loss of acoustic energy due to reflection of only 1 dB, is in a different case. The stapes and extra-stapes are rigidly connected together, so that the transformer effect is entirely due to the ratio of the effective area of the tympanic membrane and the area of the footplate of the stapes, which supports Huxley's point that the lever action of the auditory ossicles is not necessary for a high transformer ratio. But, in *Gekko*, Bárány's mechanism would not work.

Gekko must have some other device to limit background noise and damage, which may well be that provided by the stapedial muscle, the role of which in attenuating undesirably loud sounds has recently been discussed⁷. It is significant that *Gekko* is unusual in having a stapedial muscle, found otherwise in lizards only among the Pygopodidae and the amphisbaenid *Bipes biporus*⁸. In the Pygopodidae, the stapes and extra-stapes are fused together as in *Gekko*, whereas in *Bipes* the extra-stapes is absent. In those tetrapods with a tensor tympani muscle, this could also have a protective function

similar to that of the stapedial muscle⁸.

Whatever the details, there is no doubt that more attention needs to be paid, in studies of both fossil and recent tetrapod ears, of the problems of all these animals in preventing damage to the organ of Corti from excessive movement of the stapes.

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Inositol phosphate and Ca^{2+} flow

SIR—In a recent review article¹, Berridge and Irvine discuss how the phosphoinositide messenger inositol 1,3,4,5-tetrakisphosphate ($\text{Ins}(1,3,4,5)\text{P}_4$) may act as a cellular signal by modulating intracellular calcium concentrations. In reviewing the evidence in favour of the idea that $\text{Ins}(1,3,4,5)\text{P}_4$ modulates calcium influx across the plasma membrane, however, they fail to mention that the observations on which this idea was based² cannot be reproduced by the original authors³ or by others⁴.

The original suggestion that $\text{Ins}(1,3,4,5)\text{P}_4$ may gate calcium influx was based on experiments in sea urchin eggs² and attracted considerable attention^{5,6}. Crucial to the argument was the finding that egg activation required external calcium^{5,7}. This came as something of a surprise to those who routinely work with sea urchin eggs, as it had previously been shown^{8,9} that eggs and embryos fertilize and develop normally in the absence of calcium. It was less surprising, given what is known about egg activation, that the observation could not be repeated⁴.

There may be some substance in the view that $\text{Ins}(1,3,4,5)\text{P}_4$ affects calcium influx, particularly in the light of data obtained from mouse lacrimal glands¹⁰. It nonetheless seems a little unusual to find a hypothesis on results that cannot be reproduced.

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IRVINE AND BERRIDGE REPLY—We are somewhat amazed that the phrasing in our review¹ seems to have perpetrated a misunderstanding about the possible role of $\text{Ins}(1,3,4,5)\text{P}_4$ in regulating Ca^{2+} homeostasis. We have never made any attempt to disguise or play down (to the contrary^{3,11-13}) the differences that can occur between batches of sea urchin eggs. These may be due to any number of unknown factors, and a seasonal wild-animal species shipped across an ocean is always a potential source of such problems. Furthermore, there are many examples in the

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scientific literature of instances when a change in the properties (or the loss) of a cultured cell line has made it difficult to reproduce an originally highly reproducible and quantifiable phenomenon. Such circumstances do not necessarily negate the original observations — the date cannot disappear — but make it essential to look for the same phenomenon in other biological systems. By doing this, a synergism has been confirmed between $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ in controlling intracellular Ca^{2+} in lachrymal cells^{10,14}, *Xenopus* oocytes¹⁵, *Aplysia* bag cells (L. Fink, J. A. Connor, R. F. I. and L. Kaczmarek, manuscript in preparation) and permeabilized lymphoma L-1210 cells (P. Cullen, A. P. Dawson and R. F. I., manuscript in preparation).

As to the issue of a lack of dependence on external Ca^{2+} of sea urchin egg activation, this again we have agreed with^{3,13}, and the wording in our review¹ in no way implies that the $\text{Ins}(1,4,5)\text{P}_3$ – $\text{Ins}(1,3,4,5)\text{P}_4$ synergism has to be due solely to the entry of Ca^{2+} . To the contrary, evidence from lachrymal cells¹⁴, *Aplysia* and lymphoma cells indicates that $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ can also be synergistic in mobilizing intracellular Ca^{2+} . This, as we stress in the text and in Fig. 3 of ref. 1, we believe to be a principal effect of $\text{Ins}(1,3,4,5)\text{P}_4$, and Ca^{2+} entry¹⁰ is probably modulated indirectly, perhaps by a Ca^{2+} -entry mechanism controlled by a Ca^{2+} store^{16,17}. That $\text{Ins}(1,3,4,5)\text{P}_4$ controls the access of $\text{Ins}(1,4,5)\text{P}_3$ to some intracellular stores^{3,12,13}, is still only an hypothesis, but it is one founded on firm experimental fact.

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Meltwater Younger Dryas upheld

SIR—Shackleton¹ suggests that the oscillatory relaxation of ice age conditions around the North Atlantic of Broecker *et al.*^{2,3} is but a pretty house of cards which has been shaken down by a recent study⁴ of sea level and oxygen isotope chronologies at Barbados. As an early proponent^{5,6} of a physical link between runoff patterns from North America and climatically significant ocean circulation transients, I am concerned that unwarranted assumptions about the physics of the situation may colour the discussion of the isotope data. Fairbanks assumes (and Shackleton accepts) association of unique climatic states with specific runoff patterns and also that we may apply equilibrium concepts to a process whose time-scale is comparable with that on which the deep oceans respond to significant changes of circulation and composition. I shall address the first point and briefly comment on the second.

Detailed models of the global climate incorporating external forcing conditions (geography, atmospheric composition and the solar constant, for example) show that there can be several equilibrium states associated with radically different states of the global ocean circulation. This outcome of the models arises because the oceanic salinity distribution, in the absence of the explicit feedback on the atmospheric–terrestrial hydrological cycle, is extremely sensitive to the large scale oceanic transport rates.

The oceanic processes involved are shown most clearly in a model study by Bryan⁷, who found several equilibrium states in a pole-to-pole spherical sector basin (of Atlantic-like dimensions) forced by meridionally symmetric wind, temperature and freshwater fluxes. State switching in this model can be achieved by a brief transient freshwater injection. Extending this result, Manabe and Stouffer⁸ showed that coupling global oceanic and atmospheric general circulation models, with currently conventional resolution, yields a system with climatic hysteresis (intransitivity). Depending on the initial conditions, either a state like the present climate ensues, or else one bearing a strong resemblance to the Younger Dryas situation as described by Broecker *et al.* That is characterized by a blocked thermohaline circulation in the North Atlantic; once induced, it does not require a sustained anomalous freshwater source. On this model, meridional water vapour transport to high northern latitudes is somewhat less in the cold northern climate case, which undercuts a key element of the Fairbanks–Shackleton argument, but leaves us with the problem of what terminated the Younger Dryas, rather than of

what caused it.

We may have posed the wrong question. Perhaps the Allerød–Bolling phase is the anomaly, brought about by a temporary southward displacement of the meltwater output which allowed, in turn, premature enhancement of the Atlantic thermohaline circulation and an associated high-latitude warming. If so, the end of the Younger Dryas is in fact the proper termination, possibly aided by decline in meltwater drainage from the Baltic region. Attempts to resolve such questions based on oceanic paleodata are complicated by the similarity of the time-scales of the expected composition transients in the interior ocean and of the climate fluctuations, meaning that tracer signatures may be distorted by (more or less transient) global patterns of oceanic mixing and advection.

The interpretation of Fairbanks's data should also take into account influences from the Southern Hemisphere, since the tropical Atlantic surface waters are ventilated, in our present climate at least, by the southern hemisphere source of the upper thermohaline circulation branch^{9,10} (and also influenced by drainage from the Amazon and Orinoco rivers). That implies that attempts at paleodiagnostics at high time resolution (less than 1,000 years) should be undertaken in the context of global circulation simulations.

Shackleton's final challenge to Broecker, to divine another ingenious model, seems misplaced. The immediate problem appears to be not with the model, but with a misapprehension of what it must accomplish.

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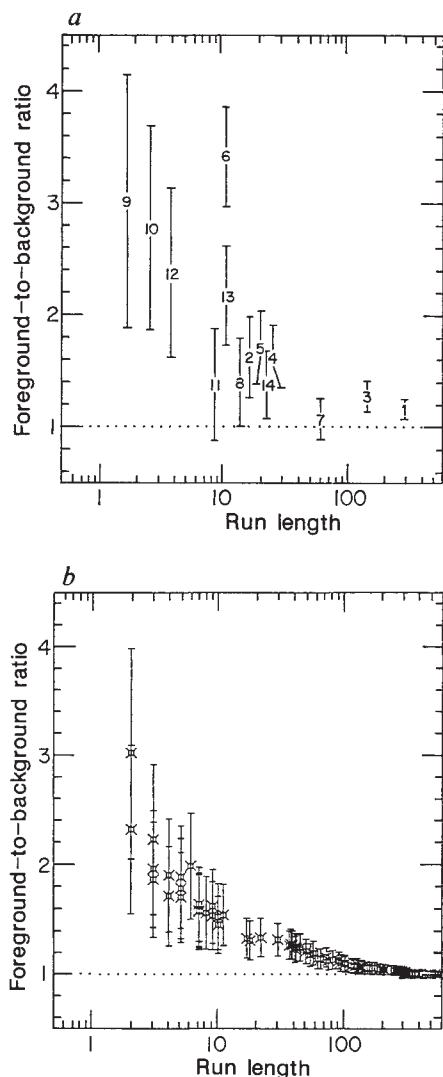
Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. They need not arise out of anything published in *Nature*. In any case, priority will be given to letters of less than 500 words and five references. □

Biases in cold fusion data

SIR—Jones *et al.*¹ have claimed to have observed 'cold' nuclear fusion. Their data, however, have a peculiar characteristic, which may indicate a systematic bias in the data collection procedure.

In Fig. 4 of ref. 1, which gives the foreground-to-background ratio from 14 different experimental runs, each of the data points indicates a positive excess,



a, Results from Jones *et al.*¹ plotted against run length. Run length is scaled by the square of the stated errors. Data points are labelled by run number. b, Results from a simulation of a biased run-length selection algorithm. Only statistical errors are shown.

which is taken as evidence for cold fusion. Runs with largest signal also have the largest statistical errors, indicating that for these runs data were collected for only a short time. Our figure *a* shows the data of Jones *et al.* for 14 runs replotted as a function of the run length. (Here we have determined the run length by assuming that the stated error is statistical.) It is evident that the data collection time varied enormously from run to run. Although the apparent systematic correla-

tion of signal rate and run length may be explained by Jones' hypothesis of a time-varying signal, there are other causes that do not require the existence of a true signal at all.

If the length of the run is chosen on the basis of information obtained from the data collected, the experimenter can exert a surprisingly strong influence on the final result. In the extreme case, the experimenter systematically stops runs when the difference between the foreground and background happens to be positive. Statistical or random systematic variations can produce this difference even when there is no real signal. Such a bias would produce a strong correlation between the deviation from the null result and the size of the statistical error. We have modelled several different scenarios for experiments affected by this bias.

The results of one of our simulations are shown in *b* of the figure. The number of events assigned to foreground and background are chosen randomly at each time step from identical gaussian distributions. The mean value of the distribution changes at each step according to a gaussian distribution with a mean of 2.2 counts per step and a standard deviation of 1.9 counts per step (this models a large random systematic variation). Values of less than zero are treated as zero. The signal (foreground minus background counts) is evaluated at each step using all the data collected for the run. The run is ended and labelled as 'successful' if any of the following conditions are met: (1) the total signal exceeds 20 counts; (2) the total signal exceeds $(4 + 0.5 \times \text{number of steps})$ counts — this ensures that there are some short runs; (3) the signal exceeds 4 counts, and the run has lasted for more than 300 steps. Runs exceeding 600 steps without meeting any of the above criteria are terminated and labelled as 'failures'.

About 75 per cent of the runs are 'successful' despite the absence of a 'true' signal. For 1,000 simulated runs, there are 15 groups of 10 or more successful runs in a row. The results for the first 100 successful runs are shown in *b* of the figure. The exact fraction and distribution of runs that exhibit positive signals depends on the algorithm used to terminate the run. Nevertheless, the general correlation between signal strength and error (or run length) is evident and reproducible for each of the six different algorithms that we have considered.

We find that statistical fluctuations alone cannot quantitatively reproduce the high foreground-to-background ratios reported by Jones *et al.*¹; but systematic fluctuations to realistic size (such as those introduced in the above scenario) may be sufficient to reproduce them. For exam-

ple, a small time-dependent gain shift in the neutron detector used by Jones *et al.*, combined with a selection bias, can lead to an apparently positive result. The shape of the observed neutron energy spectrum and the location of the expected signal peak makes gain stability particularly important.

Except for run 6, which shows the most significant variation from the null hypothesis, the results of ref. 1 do seem to follow the trend predicted for a run-length selection bias. It is impossible to prove that selection bias is the origin of the 'positive' result obtained in ref. 1, but we emphasize that it is a potentially dangerous feature of experiments in which the running time is not selected *a priori*. The presence of the characteristic correlation makes it difficult to evaluate the statistical significance of the conclusion of Jones *et al.* in the absence of detailed information about the criteria used to select the run times.

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JONES *ET AL.* REPLY—Freedman and Krakauer correctly state that by judiciously stopping a random Poisson process the experimenter can bias the count data to give an apparent statistically significant effect. The key to creating such a bias from a noise-only signal is that the decision to stop the experiment be based on the data of that run observed to date; when the decision to stop is independent of the data collected, the bias cannot be created. In our case, the decision to terminate a run (or run sequence as in the case of runs 5–7) was independent of the data collected previously during that run, and we undertook procedures to guard against systematic errors.

The first four of our foreground and corresponding background runs were of arbitrary length, determined by convenience in changing electrodes and electrolytes. The data were collected on a multi-channel analyser, without any review of the time histories of data acquisition. We did notice in the combined foreground runs the appearance of a small signal at approximately the correct energy for fusion neutrons (2.5 MeV). After run 5 had been running for 2–3 hours we noticed that the rate increased dramatically. We stopped run 5 and immediately started run 6 in order to isolate this set of data for separate analysis. When the rate decreased several hours later, we started run 7. This run was terminated at an arbitrary time, so that runs 5 to 7 had a total length that was independent of the data collected. (For run 6 we checked that emission rates of neutrons with energy

greater than ~ 2.7 MeV were the same as those for background runs whereas the count rate for 2.5-MeV neutrons was about 3.5 times greater than background.) Based on the appearance of enhanced signal lasting for only a few hours, we reduced subsequent run times to improve timing information. This accounts for the shorter runs with larger error bars noted by Freedman and Krakauer. Run 12 showed a high signal rate for about 7 hours, after which it decreased to about the background level. Analysis of the data under the assumption of Poisson counts is not biased by these run lengths provided that all runs which were broken up (such as runs 5–7) are included together.

As a further check, we have analysed separately the data from runs 1 to 7. The analysis was performed using the exact conditional test to compare two Poisson counts (background and foreground). If there is no fusion, the differences in counts, conditional on the total counts, follows a binomial distribution with the parameter P given by the total time of the foreground runs divided by the total time of all (foreground and corresponding background) runs. The actual observed number of counts is 4.4 standard deviations above the expected number under this null hypothesis; the likelihood of such an event happening by chance is $< 3 \times 10^{-5}$. These results agree with the statistical method of analysis used in ref. 1.

Freedman and Krakauer conclude that "statistical fluctuations alone cannot quantitatively reproduce" the effect reported in ref. 1. They suggest that systematic variations should be checked, which we have done carefully¹. In particular, we checked regularly for gain shift in the neutron spectrometer; this was done as follows. With an intense neutron source (^{252}Cf), we produced neutron coincidences in the ^6Li -doped glass, which gave a narrow 'glass peak' in the pulse height spectrum. This peak wandered over less than 3 channels out of 56 during the data-collection period. We then introduced electronically a shift of this magnitude, but this did not generate any structure in the difference pattern. We conclude that such drifts cannot account for the appearance of a peak centred at channel 100 with a width of about 60 channels (see figure, this page). This latter structure is, however, totally consistent with detection of 2.5-MeV neutrons, as determined by calibration with neutrons of known energy^{1,2}.

In addition, a number of control experiments were performed before ref. 1 was submitted. Light-water controls were carried out. We ran tests with a large compressor operating near the spectrometer, with a small Van de Graaf machine

producing sparks a few centimetres above the detector, and with an ultrasonic

cleaner adjacent to the detector. Our neutron spectrometer turned out to be insensitive to such noise, vibrations or arcing. We measured the background following a long exposure of the system to a ^{252}Cf calibration source and verified that no 'fake' signal was produced by material activation.

Cosmic rays are a possible source of systematic error. Cosmic-ray neutron intensities recorded at Climax, Colorado, and other stations³ show variations of ≤ 4 per cent during the data-collecting cycle (31 December 1988 to 6 March 1989), and there are no correlations with the 2.5-MeV neutron signals reported in ref. 1. We observed a very large drop in the cosmic-ray

neutron flux on 13 March 1989 which lasted several days and was correlated with solar flare activity, but this occurred after collection of the data in ref. 1. We have also checked for and excluded possible correlations of the neutron signal with solar proton events⁴. We have examined the dependence of cosmic rays on barometric pressure. The underground laboratory at Brigham Young University lies below approximately 2 m of soil and 15 cm of concrete. Using data from ref. 5, we estimate the barometric coefficient for cosmic-ray neutrons in our laboratory to be only -0.2% mbar⁻¹. We verified that there is no correlation between barometric pressure variations and the appearance of 2.5-MeV neutron signals reported in ref. 1.

With regard to systematic errors, we note that experiments performed in collaboration with researchers at the Los Alamos National Laboratory show that neutron bursts occur with highest probability in partially deuterated titanium alloys when the sample temperature is approximately -30°C (ref. 6). This temperature correlation greatly reduces the likelihood of systematic error and suggests paths to better understanding and reproducibility of neutron production in partially deuterated metals⁶.

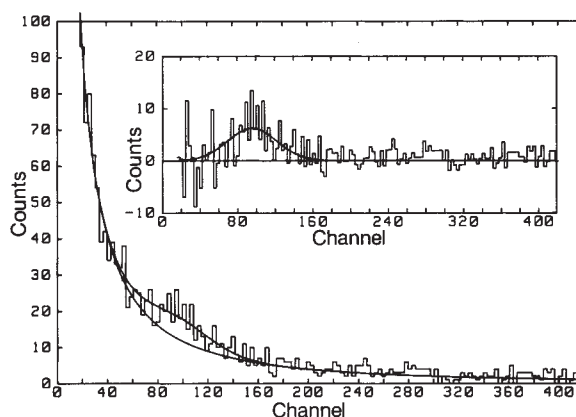
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Reanalysis of the original data set of ref. 1. This method of analysis illustrates the merit of using an energy-sensitive neutron spectrometer. Adding together all background data, we have fitted the summed background distribution from channels 16 to 420 inclusive, at three channels per bin, with the function $y = A/(x^2 + Bx + C)$, where x is the bin number and y represents counts in bin x . The values of B and C vary the shape of the curve, and A represents an amplitude or scaling factor. Channels 1–15 are ignored because of the artificial cutoff at low energies; the long high-energy tail is also truncated. The values that give the best least-squares fit to the background data are: $A = 61,100 \pm 4,600$, $B = 46.2 \pm 4.9$ and $C = 198 \pm 17$. The value of χ^2 for the fit is 137 with 132 degrees of freedom, indicating an excellent fit to the background data (see ref. 2 for further details). A monoenergetic neutron signal should form a roughly gaussian structure above the background, according to calibration studies², so we fitted the sum of all foreground data¹ (binned as before) with the function

$$y = \frac{A}{x^2 + 46.2x + 198} + D \exp \left[-\frac{(x - p)^2}{w} \right]$$

where we kept the background-shape parameters B and C constant. D , p and w are the amplitude, centre and square of the width of the gaussian, respectively. The best nonlinear fit yielded a χ^2 value of 103 with 131 degrees of freedom, an excellent fit to the data. We obtain $A = 27,290 \pm 870$, $p = 27.3 \pm 5.2$, $w = 152 \pm 50$ and $D = 6.1 \pm 1.5$. The statistical significance of the peak is greater than 4σ , including correlations between the parameters. The centre of the gaussian is at channel 97 ± 16 with a full width at half maximum of 60 ± 11 channels. Both the position and width of this feature are consistent with those expected for 2.5-MeV neutrons detected in the BYU neutron spectrometer, based on calibrations with neutrons of known energy^{1,2}. This result adds to our confidence that the feature represents neutrons generated by fusion of deuterons. We cannot distinguish clearly in these data whether the neutrons were produced randomly or in bursts⁵. By examining the correlation coefficient between the parameters A and D , we find that a 5σ increase in the background would be required to remove the significance of the gaussian peak.

... nor any drop to drink

Roland Bailey, Tony Bark and Roger Miles

Biology of Wastewater Treatment. By N.F. Gray. Oxford University Press: 1989. Pp. 828. £70, \$80.

Nitrates: The Threat to Food and Water. By Nigel Dudley. Green Print, an imprint of Merlin Press, 10 Malden Road, London NW5 3HR, UK: 1990. Pp. 118. Pbk £4.99.

THIS year marks the end of the "decade of international drinking water supply and sanitation", a campaign to improve the lot of less well-off countries. During the same period, these services, long taken for granted, have assumed a high profile in Britain as a consequence of the privatization of the regional water authorities in England and Wales, coupled with publicity stemming from accidents in treatment plants, overloaded sewage works, crumbling sewers and the failure of water supplies and bathing beaches to meet European Community standards. Seemingly against the wishes of the majority, the water utilities joined the private sector with considerably oversubscribed share issues. Pollution problems and demands for environmental protection remain, however, and there is scepticism among the general public about the ability of the reshaped industry to deal with these issues.

On the plus side, an important accompaniment of privatization is the separation of the 'gamekeeper', in the form of the National Rivers Authority, from the 'poacher'. The National Rivers Authority is a public body set up to regulate water resources, including conservation and recreational aspects. Early anxieties about the authority, expressed by a 'not required any more' syndrome apparent among some scientists who used to work for the regional water authorities, have given way to very positive attitudes, from the acquisition of independent facilities to additional recruitment. The National Rivers Authority has already prosecuted parental bodies and other polluters. It is to be hoped that this vigilance will continue and that proposals for monitoring and research into environmental improvements will not be frustrated by financial constraints.

The main concern for the utilities must be money, to improve or renew ageing

plants, water mains and sewers, and for research and development. As well as increased charges to consumers and consultancy earnings, there will undoubtedly be pressure to add to investment income

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REASONS

by selling land that is not used for water services. Fears about under-investment in sewage treatment and disposal, which does not generate direct profits, should be allayed by the realization that to obtain sufficient quantities, the reuse of available water is essential and the upsurge in legal and public demands for environmental protection will not diminish. In these early days, the water industry is adding to its scientific staff, who have important roles in the monitoring and optimization of operational processes and in the design of the next generation of treatment systems.

An increased demand for scientists is encouraging to those, like N. F. Gray, involved in their training. Gray's book, a set text for MSc courses in environmental science and engineering, reviews waste waters, natural pollution control and treatment options. It contains an excellent critical description of microbial oxygen demand and its measurement, but the

discussion of the role of organisms is of variable quality. A good summary of their variety and ecology contrasts with some laxity in sections on stoichiometry, kinetics and metabolism. Statements such as "the citric acid cycle is intermediate between anaerobic and aerobic metabolism" are likely to confuse, and more care might have been given to the diversity of fermentation pathways, bacterial electron-transport chains and ATP yields from metabolism.

A long chapter on biological treatment processes incorporates process controls, engineering procedures and costings in very thorough accounts, including less well-known variants, of percolating

filters, activated sludge, anaerobic unit processes and 'natural' treatment systems. Sludges are an unwanted product of wastewater treatment which receive a bad press, as although there is no provably safe way to dispose of these complex materials, they cannot be stockpiled. Gray offers a well-balanced review of the relative advantages and risks of land and sea disposal. His analysis of the impact of metals, nutrients and other contaminants, especially in the North Sea, is a useful perspective in view of attempts to control sludge dumping.

Public health aspects receive anticipated coverage, but Gray's concluding chapter on biotechnology is an exciting surprise. The applications of biotechnology range from resource reuse, bio-energy and single-cell protein production to environmental protection using packaged microbes. There are enjoyable references to the advantages of the family pig to ensure sufficient wastes for the biogas needs in Chinese homes, and to the purchase of packaged bacteria to degrease sewers beneath New York's restaurants. As these examples show, although Gray is chiefly concerned with Europe, he makes frequent excursions into the global scene.

The breadth of this volume is a remarkable undertaking for one author. The result contains a significant number of minor errors, ambiguities and repetitions, but these detract very little from the overall value of the book. It is impressively referenced, the full list occupying 85 pages, with key works indicated for each section. Gray's optimistic prognosis is that with the evolution of new, more efficient and compact treatments, there will be a

shift of emphasis from traditional aims towards environmental protection linked with resource conservation and by-product recovery which, in turn, will offset operating costs.

Although slender by comparison, Dudley's book merits attention as raised nitrate levels are one way in which European Community standards for drinking water have been increasingly breached in Britain. Moreover, the significance of nitrates in food and water is an issue on which researchers disagree, vested interest looms large and public suspicions run high. Dudley carefully presents information on nitrate entry into diets and the putative link between nitrate, nitrosamines and cancer, and punchy sections on "kicking the nitrate habit" consider the

costs, politics and methods for reducing nitrates in drinking water, food and the environment. (Curiously, options for nitrate removal receive scant treatment by Gray.)

The chemistry given by Dudley, however, would have benefited significantly from rigorous scientific editing; he has a general and confusing tendency to refer to any form of nitrogen as nitrate. This reservation apart, the extensive up-to-date source material will make this a useful popular science book, addressed to the concerned layman rather than the informed specialist. □

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Summing up

Ian Stewart

The Development of Newtonian Calculus in Britain 1700–1800. By Niccolò Guicciardini. *Cambridge University Press*: 1989. Pp. 228. £35, \$54.50.

WHAT happened to mathematics in Britain in the period after Newton? It is often held that the subject entered a lengthy period of decline, caused by an obsession with the newtonian approach to calculus and an unwillingness to follow the continental usage of Leibniz. Newton's notation for differentiation was to put a dot above the appropriate symbol, and this period is therefore referred to as the "dot-age" of British mathematics.

Is this a fair picture, or is the dot-age a myth? To answer these questions, Guicciardini takes a careful and informed look at what actually happened when British mathematicians took up Newton's ideas. The first part of his book describes how calculus began to diffuse into public consciousness, mostly through the medium of lectures. It tells how the calculus itself was developed by people such as Brooke Taylor, James Stirling and Colin Maclaurin. It examines the controversy over the logical foundations of calculus that came to the boil in 1734 with the celebrated criticisms by Bishop Berkeley.

The second part describes the flood of textbooks that followed these criticisms, the widespread interest in mathematics among amateurs, and the flourishing of mathematical societies. We read of the work of Colin Maclaurin on the attraction of ellipsoids and John Landen's anticipation of some results on elliptic integrals. The third part is devoted to the attempts to reform the calculus that were made around 1800, initiated by John Playfair and taken up by William Wallace and James Ivory in

Scotland and John Brinkley in Ireland.

Finally, the author sums up his conclusions. A crisis did occur, but it set in later than is usually imagined. Lack of contact with continental mathematics was more to blame than adherence to any particular symbolism. Throughout the decline, many mathematicians were aware of the problem and fought to put it right. Eventually, this school of thought triumphed; then to some extent it rewrote history and the myth of the dot-age was born.

The author perhaps places a little too much value on the achievements of eighteenth century British mathematicians in developing calculus. Some good work was done, contrary to the myth; but it pales into insignificance compared with the contributions of Euler, Lagrange, Laplace, the Bernoullis and the hordes of continental mathematicians who made the subject the core of mathematical physics. The dot-age may be a myth, but the myth contains more than a grain of truth.

The book, which is documented in great detail, will be of value to anyone interested in this particular period of British mathematics. It also provides a much-needed reminder that mathematical ideas are transmitted through teaching and cultural influences as well as through research. □

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■ Also recently published by Cambridge University Press, *The Preliminary Manuscripts for Isaac Newton's 1687 Principia, 1684–1686* comprises photo-facsimiles of Isaac Newton's manuscripts originally published in 1687. The manuscripts are selected from the unmatched collection of Newton's writings in Cambridge University Library. Newton's *Principia* are considered to be the most influential work that has appeared in the field of mathematical physics and astronomy. In this volume, the manuscripts are annotated by D. T. Whiteside. Price is £60, \$110.

Causes and consequences

Simon Wain-Hobson

Retrovirus Biology and Human Disease. Edited by Robert C. Gallo and Flossie Wong-Staal. *Dekker*: 1990. Pp. 409. \$99.75.

HERE is yet further confirmation that human retrovirology is a most complex and fascinating field. I used to think, having worked on hepatitis B virus, that HBV had everything. Yet the human immunodeficiency viruses (HIV) are just that much more subtle. (I shouldn't quibble as both are, after all, retroviruses of sorts.) In the 400-odd pages of *Retrovirus Biology and Human Disease*, we are swept through animal and human retroviruses, molecular biology, epidemiology, pharmacology and, of course, clinical AIDS. The editors aim to provide a picture as complete and as broad as possible of the mediagenic yet frightful disease AIDS.

The plan of the book is not surprising, given that Robert Gallo is an editor. The book starts with a historical overview and continues with a walk through bovine and feline leukaemias and their retroviruses, in particular the feline virus, which causes more immunosuppression than leukaemia. These viruses were among the first retroviruses found to be involved in real (out of the laboratory) disease. They pointed the way, even though it was long, to the human T-cell leukaemia viruses (HTLVs). In some ways, the casualty of the search for oncogenic human retroviruses were the ungulate lentiviruses which caused catastrophic sheep loss in Iceland during the 1930s and 1940s. As it happened, the AIDS (or immunodeficiency) viruses turned out to be the first human/primate lentiviruses discovered. It is therefore appropriate, and comforting, to see the ungulate lentiviruses included in a book on human retroviruses.

Adult T-cell leukaemia/lymphoma, its epidemiology and its virus are dealt with next, followed by HTLV-II. This last is an odd fellow in that there are so few cases of HTLV-II. But, sadly, a recent apparition among intravenous drug users means that we are going to see more of this virus.

HIV is extensively covered, from the fine minutiae of the *vpu* gene to problems of treatment by the drug AZT and of vaccines. The chapters on this virus are rather mixed, ranging from vistas to laboratory results. HIV-2 is treated as a HIV-like virus, which will serve only to confuse. The simian viruses get short shrift, which seems unfair as they represent the only way to test hypotheses as to precisely which genetic locus or loci are

important to the induction of AIDS. Furthermore, these viruses are central to vaccine development, particularly given all the difficulties of working with chimpanzees.

The book is not as homogeneous as could be desired. There are a few chapters which may be digested by the seasoned retrovirologist but will pose problems for others. The other difficulty is the explosion in the field since the discovery of HIV-1: in the space of only six years, seven primate or monkey viruses and a cat virus have been discovered, and bovine immunodeficiency virus has been resurrected. The field is expanding so rapidly that nobody can spare the time to update

the standard reference work, *RNA Tumour Viruses*, either because the task is so daunting or for fear of being out of date as soon as the book hits the press. Even conference volumes are often too late. This one is about on target but is bound to be *depassé* within a year or so. Already there are important omissions. Perhaps the only solution is to produce frequent reviews and volumes like this one. Yet this very problem only confirms my belief that this is a fascinating field. □

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again."

See what I mean? The story is the stuff of an unparalleled romantic epic novel. And Rosalynn Pflaum sensibly exploits its possibilities in this very readable, and well worth reading, biography. But the story is not a novel — although as Richard Ellmann has pointed out, modern novels have led us to expect to see the worst in the subjects of biographies as well as the best. And this brings us to the book's weakness — none of the characters seems to have a worst. Irène may have been a bit surly at times, but that's about the limit. Marie may have seen more than was strictly prudent of the married Langevin, and here a real scandal ensued. But the relationship seems to have been more the occasion for it than a real cause. Was Frédéric a cynical opportunist and philanderer — the dark side of his cleverness and charm? I never found out.

Does the book throw any light on the nature of science? It certainly describes some of it ably enough. A little while ago, David Hull reviewed the new edition of *The Selfish Gene* by Richard Dawkins (*Nature* 342, 319–320; 1989). Hull wrote,

Nuclear family

John Galloway

Grand Obsession: Madame Curie and Her World. By Rosalynn Pflaum. Doubleday: 1989. Pp. 496. \$22.50.

I DEFY any romantic novelist, alive or dead, to have plotted a better story than that of the Curies — who I suppose were the original nuclear family. Marie Curie began life as (variously) Marya or Manya (as here) Skłodowska in mid-nineteenth century Poland, a country crushed under the Czarist heel. Brought up in a free-thinking, exciting, liberal home she later supported her sister Bronya's medical studies by acting as governess in the intellectually stultifying household of the supervisor of a sugar-beet factory. She fell in love with the dashing son of the house, but it was not to be. He needed a better marriage, one to keep him in the style to which he intended to become accustomed, than one to clever — but poor — Manya.

Then on to (relative) poverty and academic obscurity as a student in Paris. Marie's ambition was resisted all the way by the intensely snobbish, conservative and militantly anti-women French scientific establishment. But she married the brilliant Pierre Curie and together they won the Nobel prize for physics. Eight years later she won the Nobel prize again, this time for chemistry — and this time on her own.

On to the next generation. Daughter Irène married clever, charming Frédéric Joliot, who may or may not have had an eye for the main chance in marrying into the by now world-renowned Curie dynasty. In 1935, the couple repeated the triumph of the earlier generation by winning the Nobel prize yet again — for their discovery of artificial radioactivity. Irène and Frédéric played a major part in defeating Germany in the Second World War, by persuading the French government to buy up stocks of heavy water. And

Frédéric went on to head the French Atomic Energy Commission.

But there is a more darkly ironic and tragic side to the tale. All four Curies fell victim to the very success of their research. Pierre tripped and fell under a dray and his head was crushed, an accident very probably caused by absent-mindedness brought on by breathing

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

A family apart — Marie Curie with her husband, Pierre, and their oldest daughter, Irène, in 1904.

radioactive radon. Marie and Irène probably both died of leukaemia induced by radiation. Frédéric went one better. Dismissed from his post because of his politics — he was an ardent communist and the Americans didn't like that — he died of cirrhosis of the liver, probably caused by polonium toxicity. A nice symmetry there, Poland starting the Curies' story and ending it. But Frédéric was vindicated. Charles de Gaulle wrote his epitaph "I am the one who made him head of the Atomic Energy Commission. I am very satisfied with what I did and I would do it

"scientists give every appearance of being addicts and science is their vice". In the addiction stakes, Marie was a 200-a-day woman. Not for her a fellowship at a comfortable Oxford college; she worked 12 hours a day in a leaky hangar in the back yard "in conditions that no self-respecting heavy labourer would have tolerated". And she worked for nothing. That's what I call addiction. And, of course, one of the salutary messages the book provides is how an addiction for the esoteric ideas of atomic physics led so rapidly to their becoming influences that

shaped world history, for better or worse.

Given its subject, the book has to provide insight into the ever-topical question of women in science. Women have not often won Nobel prizes. Of 566 laureates up to and including 1988, it looks as though only 19 have been women (although I am open to correction on this — very properly in this feminist age the *Nobel Foundation Directory* does not include the sex of the winner). Furthermore, Marie Curie is the only woman ever to have won the prize for physics, and of the eight science prizes awarded to women, the Curies have won three. Only four people have won the Nobel prize more than once — Marie Curie, John Bardeen, Fred Sanger and Linus Pauling. Marie was the first woman to be awarded any Nobel prize and no woman other than a Curie had won the prize for science until Dorothy Hodgkin in 1964.

By contrast to art and literature, success in science presumably usually demands some sort of public position and command of resources. There is no doubt, as the book makes clear, that in the early part of

the century women were scarcely tolerated in science. That there are precious few around today finds its roots in this. The problem did not exist simply in France, but also in Britain and Germany, where Max Planck said "If a woman has a special gift — which does not happen very often, I do not think it right to refuse her the chance to study..., but such a case must be regarded as an exception." And Planck was among the most progressive of his generation. Marie Curie's friend, Hertha Ayrton, was refused a fellowship of the Royal Society apparently because she was a married woman. But the trouble is that the Curies are too extreme to be a useful example. They test to destruction any possible sociological theory for the absence of women in science.

Anyone who turns winning the Nobel prize into a family business deserves a biography. The Curies were the family. This is the biography. I think it is rather good. □

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Not so sweet dreams

John Walter

Poisons of the Past: Molds, Epidemics, and History. By Mary Kilbourne Matossian. Yale University Press: 1989. Pp.190. £18, \$25.

Bread of Dreams: Food and Fantasy in Early Modern Europe. By Piero Camporesi. (Translated by David Gentilcore.) Polity: 1989. Pp.212. £19.50, \$27.50.

THE past, we are told, exists only in the present. If so, then these books dealing with the historical impact of contaminated foods are timely reminders of the exchange between the concerns of the present and the past in the construction of history. Both deal with past societies, for which the invocation "give us this day our daily bread" had a special resonance, since the consumption of bread in large quantities was for most their daily diet. The authors of both these books argue that the tainted nature of this bread killed in appalling numbers and, more provocatively, that it left many in a quotidian world of narcosis and neurosis.

Demographers have become increasingly aware of the central importance of disease in understanding past demographic regimes. Matossian argues that they need to add an awareness of the significance of mycotoxicoses in explaining the behaviour and, she argues, beliefs of past societies. Central to her thesis is the prevalence of ergotism in past

societies where rye provided the main foodstuff.

Ergotism in rye can be lethal. In Russian epidemics of the nineteenth century, mean mortality rates of those reported affected were just over 40 per cent. In addition, ergonavine can act as an abortifacient whereas other alkaloids suppress fertility or lactation. Moreover, by acting as immunosuppressants, these chemicals weaken the body's resistance to infectious disease. Matossian claims to find in these relatively recent discoveries answers to some of the conundrums that have previously puzzled demographers. If a population failed to grow after a plague, and renewed epidemics do not explain why, then try ergotism, she suggests. Similarly, ergotism (and fusarium moulds on poorly stored grain) helps to explain peaks in mortality and troughs in fertility in successive centuries. Matossian suggests a switch from rye to wheat, maize and potatoes as staples of people's diet to explain that final demographic conundrum — the take-off of western Europe's population into self-sustaining growth.

Arguing that there was a similar improvement in the mental health of Europe's population after 1750, Matossian goes on to claim a role for ergot poisoning in explaining what she believes to be past collective psychoses. Ergot, a source of lysergic acid diethylamide, may include natural alkaloids that act like LSD, creating hallucinatory and suggestible mental states, whereas other ergot alkaloids interfere with the activity of dopamine in the body, causing muscular spasm as well as delusions and hallucinations. The behaviour in the witch-craze of the sixteenth

and seventeenth centuries, the "great fear" that mobilized peasant communities in the French Revolution or the religious revivalism in the United States in the 1740s owes something, Matossian alleges, to ergotism.

This is a bold book with a stimulating thesis. Matossian's claims for the role of food poisoning will need to be incorporated into any satisfactory account of past demographic trends. Readers unfamiliar with existing work in this field should be warned, however, that the claim for the importance of ergotism is greatly helped by Matossian's tendency to dismiss too quickly existing alternative explanations and that, in the absence of hard evidence, the thesis rests on creative, but inferential, arguments that some, perhaps most, will find unsatisfactory. The argument also requires that rye was the main bread of the people, a claim that needs greater justification for England, at least. Matossian begins with good advice about the dangers of riding a hobby horse, but on occasion abandons caution. In particular, her final chapter, which seems to suggest that the key episodes in the European past (for example, the Reformation) might owe something to ergot poisoning, calls to mind the British MP Edwina Currie's disastrous courting of publicity by provocation.

Bread of Dreams covers similar territory, but from a slightly different perspective. Camporesi, a literary historian, examines the fears and fantasies Italians in pre-industrial times wove around food and famine. He uses essentially literary sources — but what sources! The writings of Baroque doctors and thinkers provide a point of entry into a world of repeated famines and persistent malnourishment where 'men of science' wrote tracts advocating an alarming range of food substitutes and believed that natural magic could convert poisonous herbs into the staff of life.

The reality, Camporesi argues, was very different. For him, Italy until at least the end of the eighteenth century was a society in which many of the people were the victims of hunger, producing hallucinations and tremors dementia. Poverty led to the baking of bread adulterated with substitutes such as poppy seed or dandelion which induced narcosis, while a peasant pharmacopeia featuring such items as henbane or hemp fibre also induced a 'green deliria'. The literary nature of Camporesi's sources makes it difficult to assess how far what he describes was an historical reality, but *Bread of Dreams* is a remarkable and exciting recovery of an earlier cosmology elaborated around bread and body. □

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Coherence established between atmospheric carbon dioxide and global temperature

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The hypothesis that the increase in atmospheric carbon dioxide is related to observable changes in the climate is tested using modern methods of time-series analysis. The results confirm that average global temperature is increasing, and that temperature and atmospheric carbon dioxide are significantly correlated over the past thirty years. Changes in carbon dioxide content lag those in temperature by five months.

DURING the past century (see, for example, refs 1, 2), scientists have studied the possibility that the climate is influenced by changes in the atmospheric concentration of CO₂ caused by industrial and agricultural activities³⁻⁵. Recently, because of the potentially serious consequences of the greenhouse effect^{6,7}, the problem has been studied more intensively^{8,9} with a view towards observing climatic effects attributable to the increase of atmospheric greenhouse gases. For example, the output of numerical models of the global climate has been compared with measurements^{10,11}, and the significance of the temperature increase has been tested using various straight-line segment and parametric models (ref. 12 and J. Seater, manuscript in preparation). But present numerical models of the atmosphere are crude, and comparisons between the time series representing the real data and predictions of the atmospheric models are difficult to interpret. Because the available data are short time series, conventional statistical methods are unreliable, and detection of the greenhouse effect remains controversial¹³⁻¹⁸.

The longest modern series of precise CO₂ concentration measurements begins in March 1958 and consists of monthly values collected by Keeling at the summit of Mauna Loa in Hawaii¹⁹. Although these data are from a single station, they are typical of measurements made since 1974 at several sites²⁰. Because we are interested in the time-series aspects of the problem, we use the longer Keeling series. The data have an upward trend that is readily visible (the upper curve in Fig. 1), an obvious annual component and irregular fluctuations. Five missing values were interpolated using a stochastic least-squares procedure on the residuals from a quadratic polynomial plus the first five annual harmonics. These interpolated values have an estimated error of 0.35 p.p.m. and are noted in Fig. 1.

Hansen and Lebedeff²¹ created a time series of monthly global-average surface-air-temperature changes from January 1880 to December 1888. This series is a weighted average over stations, formed by subtracting the average January temperature during the reference period 1951-80 from all the January data, and repeating this for the other months, eliminating seasonal variations. Displayed as the lower curve in Fig. 1, the temperature series also increases with time but its fluctuations are relatively larger than those in the CO₂ record.

Here we apply multiple-window time-series methods (which are efficient for short series) to estimate the trends and power spectra of the Hansen-Lebedeff average global surface temperature series and Keeling CO₂ concentration measurements as well as the coherence between the two. This analysis shows that from 1880 to 1988, the average global temperature increased by 0.0055 ± 0.00096 °C yr⁻¹, and the probability that this slope

is positive exceeds 99.99%. Furthermore, the monthly CO₂ concentration and global temperature series from 1958 to 1988 are coherent over much of the Nyquist frequency band from 0 to 6 cycle yr⁻¹; the probability that the level of coherence observed from 0 to 2 cycle yr⁻¹ occurred by chance is $\sim 2 \times 10^{-6}$. Not only do both series have increasing trends that are highly significant, but there are linear relations between many of their oscillatory components. We interpret this as evidence that the changes in atmospheric CO₂ concentration are closely related to changes in global temperature.

Models

Many of the contradictions in the literature about the analysis of climate data can be traced to the use of inappropriate or oversimplified models (for review, see, for example, refs 22, 23). Statistical techniques that are vulnerable to difficulties include those based on parametric models (such as low-order autoregressive moving-average representations) and methods plagued by more insidious problems caused by implicit assumptions of time-series stationarity. The cavalier use of parametric models can lead to misspecification difficulties²⁴ because

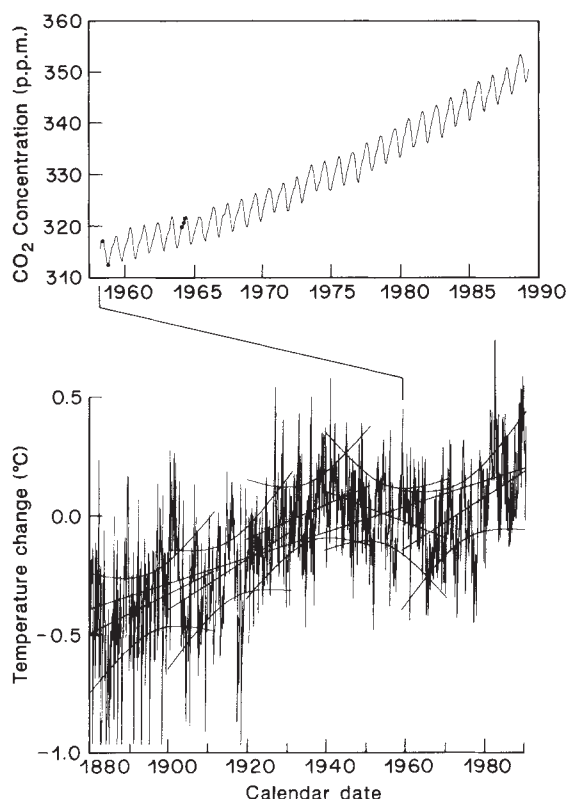


FIG. 1 The upper curve is the monthly Keeling CO₂ concentration data for March 1958 to December 1988. The five interpolated points are marked. The lower curve is the Hansen-Lebedeff average global temperature series for 1880 to 1988. The short line segments and hyperbolic arcs define trends and 95% confidence regions over 30-yr intervals, and the long straight line shows the general trend.

unanticipated effects (such as the modulation of the annual cycle discussed below) either are undetected, are attributed incorrectly or are simply lumped in as part of 'residual variance'. As shown below, the CO₂ concentration and global temperature series have complicated spectral density functions, so these series are not likely to be modelled by any simple form in either the time or frequency domains. The fundamental flaw of parametric models, including numerical atmospheric models and time-series models, is that they make no provision for the unexpected.

We represent each time series by the less restrictive decomposition into a parametric trend and a non-parametric residual. Because one cannot directly discriminate between the many possible functional forms for the trends from such short records, we represent the trend in each time series by the simplest non-constant function of time, a straight line of non-zero slope which can be thought of as the first two terms in the Taylor series expansion of the true trend. It may be argued that this semi-parametric representation is subject to the misspecification problems mentioned above, but, because we subject the residuals to greater scrutiny than we do the trends, features not included in the trends will still be present and studied in the residuals. Moreover, we find that including quadratic terms does not change the results of the following analysis significantly.

Therefore, we express each time series

$$\{x(0), x(1), x(2), \dots, x(N-1)\}$$

as the sum of a constant, a linear trend and a stationary time series specified only by its spectral representation

$$x(t) = a_0 + a_1(t - t_{\text{ref}}) + e(t); \quad t = 0, 1, \dots, N-1 \quad (1)$$

where t_{ref} is a reference time, a_0 and a_1 are constants and the residual time series $e(t)$ has the spectral representation

$$e(t) = \int_{-1/2}^{1/2} e^{i2\pi ft} dX(f) \quad (2)$$

for all t . $dX(f)$ is the differential of a generalized Fourier transform and is known as an 'orthogonal increment process'. (This is an extension of the representation used in ref. 25, where the CO₂ series was decomposed into a trend, an annual component and a residual.) The annual component is given by the first moment of $dX(f)$, and the power spectrum, or power spectral density, $S(f)$, of the residuals is, by definition, the second moment of $dX(f)$:

$$S(f) df = E\{|dX(f)|^2\} \quad (3)$$

where E is the statistical expected-value operator. In a stationary series, values of $dX(f)$ at distinct frequencies are uncorrelated²⁶.

Estimation

Although the time- and frequency-domain representations of time series are formally equivalent²⁷, we usually find it more informative to analyse time series in the frequency domain where the effects of different physical processes can be easier to distinguish. With short time series, such as the CO₂ and global temperature records, it is difficult to resolve different frequencies and simultaneously obtain statistically significant results. Our approach is to use a variant of the multiple-window method of spectrum analysis^{28,29} that, although it does not eliminate all the problems associated with short series, makes statistically efficient use of the available data.

Most frequency-domain methods are strictly valid only for the analysis of stationary data, not series with embedded trends such as the CO₂ and global temperature records. Using the multiple window procedure described below, we estimate the trends in each series, subtract off these terms, and estimate the spectrum of the residuals. Finally, we test the residuals for stationarity to see if our assumptions were violated.

Estimating the average a_0 and trend a_1 by ordinary least squares can produce misleading results if the residuals $\{e(t)\}_{t=0}^{N-1}$

have a non-white (non-flat) spectrum and are therefore correlated^{30,31}. The residual spectrum need only be roughly flat in a small frequency interval around the origin for multiple-window regression, however, to produce valid estimates of a_0 , a_1 , the spectrum of the residuals and their errors.

Multiple windows

The multiple-window method uses the orthogonal sequences of N elements that optimally concentrate the spectral energy in a frequency band of width $2B$ centred on a frequency f , that is, between the frequencies $f-B$ and $f+B$. These sequences are the lowest-order $[2BT]$ discrete prolate spheroidal sequences of ref. 32, where T is the duration of the observed series. These 'Slepian sequences' form a basis on which the data in the

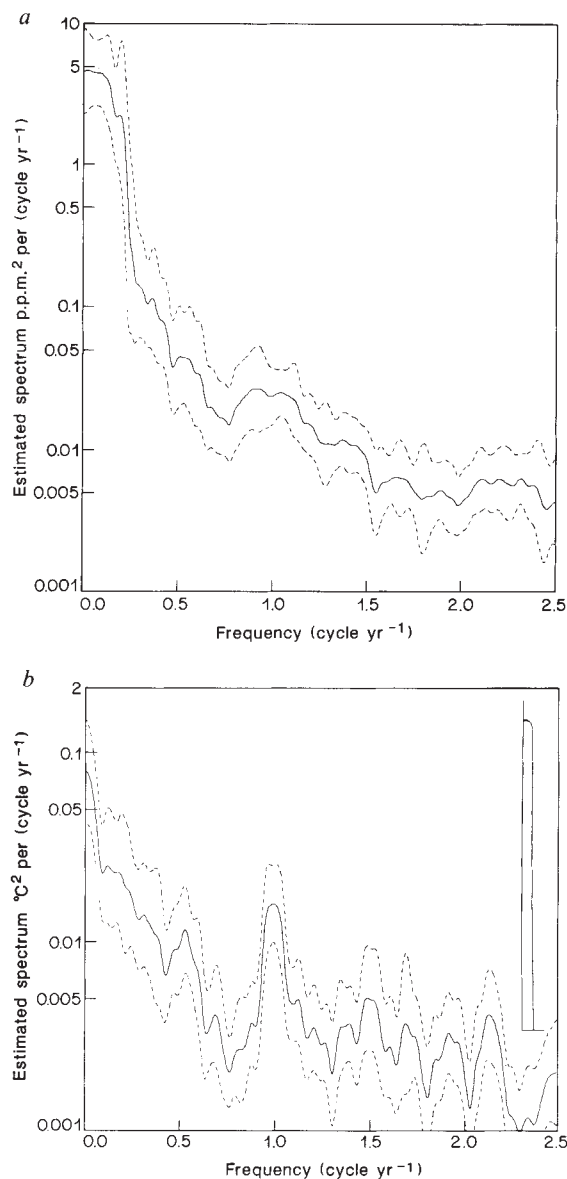


FIG. 2 *a*, Multiple-window spectral estimate of trend-subtracted CO₂ concentration series. The upper and lower dashed curves are 5 and 95% confidence limits obtained by jackknifing on the windows. Harmonics of the annual cycle have been subtracted. *b*, Multiple-window spectral estimate of detrended global temperature series for the interval 1880 to 1988. The dashed lines give the 5 and 95% confidence intervals as in *a*. The insert on the upper right shows the effective spectral window on the same amplitude and frequency scale. Note that the power around 1 cycle yr⁻¹ is not a periodic component. (Spectra done at higher resolution show that this feature is asymmetric with more power on the lower sideband than on the upper, indicating combined amplitude and phase modulation of the annual cycle.)

frequency band can be expanded. The coefficients of this expansion depend on frequency and are obtained by taking the discrete Fourier transform of the product of the data with each Slepian sequence. The lowest-order coefficient is similar to a direct spectrum estimate using a conventional data window or taper. A multiple-window estimate of the spectrum is, however, an adaptively weighted average of the $2BT$ frequency-dependent coefficients.

Because of their energy-concentration properties, the Slepian sequences are the data windows that are most resistant to spectral leakage²⁸. For example, the estimate of the temperature-series spectrum in Fig. 2b is produced using 11 Slepian sequences with $2B = 0.11$ cycle yr⁻¹; sidelobes of the effective spectral window shown in the insert are unobservable on the scale of Fig. 2b.

Choosing the parameter B for a multiple-window estimate of a spectrum involves a tradeoff between resolution and variance. The variance of this spectrum estimate is proportional to $1/(2BT)$. Thus, increasing B improves statistical reliability but decreases the resolution of the estimate.

Trend estimates

To estimate the coefficients a_0 and a_1 in equation (1) by conventional time-domain regression, one minimizes the sum of the squares of the residuals $\sum_{t=0}^{N-1} e^2(t)$ with respect to the coefficients. By Parseval's formula,

$$\sum_{t=0}^{N-1} e^2(t) = \int_{-1/2}^{1/2} |\tilde{e}(f)|^2 df \quad (4)$$

where $\tilde{e}(f)$ is the discrete Fourier transform of the residuals $\{e(t)\}_{t=0}^{N-1}$, so minimization in either the time or frequency domain is equivalent. To avoid the energy associated with the harmonics of the annual cycle and other high-frequency processes, however, we minimize only the integral over the low frequencies

$$\int_{-W}^W |\tilde{e}(f)|^2 df; \quad W = BT/N < \frac{1}{2} \quad (5)$$

and ignore the higher-frequency components of the residuals, resulting in estimates expressible in terms of the Slepian sequences.

The multiple-window approach has several advantages when the residuals are autocorrelated. First, the 'observations' in the multiple-window regression are closer to independent gaussian random variables than the original time-domain data $\{x(t)\}_{t=0}^{N-1}$, and therefore the multiple-window coefficient estimates are closer to maximum-likelihood estimates than are the estimates from ordinary least-squares analysis. Similarly, the number of degrees of freedom and error estimates are easily calculated using the Slepian sequences, and the multiple-window method is often more statistically efficient than ordinary least-squares analysis (C. Lindberg, manuscript in preparation). In conventional regression, the endpoints of the time series (for example, the abnormally high temperatures of the last decade) can have an inordinately large influence on the coefficient estimates³³. This effect is largely eliminated in this approach. Finally, it is relatively easy to check the assumptions that have been made in representing the data by a particular model.

Trends

For the monthly Keeling CO₂ series (March 1958 to December 1988, $T \approx 29.8$ yr), estimates of the average and trend were obtained using $t_{\text{ref}} = 1975.0$, and a bandwidth of $2B = 0.39$ cycle yr⁻¹. This value of B confines the spectral energy associated with the trend components to frequencies f with $|f| \leq 0.195$ cycle yr⁻¹, avoiding energy associated with the annual cycle and its harmonics. This value of B also results in a spectrum that is locally white in the resolution band (at frequencies above 0.2 cycle yr⁻¹, the CO₂ residual spectrum drops rapidly, as shown below). The time-bandwidth product

$2BT \approx 11.6$ gives eleven leakage-resistant windows. The multiple-window procedure results in the estimates $a_0 = 331.9 \pm 0.44$ p.p.m. and $a_1 = 1.191 \pm 0.053$ p.p.m. yr⁻¹.

For the monthly Hansen-Lebedeff global temperature series from January 1880 to December 1988, we obtained estimates of the average and trend using $t_{\text{ref}} = 1934.5$ and a resolution bandwidth of $2B = 0.128$ cycle yr⁻¹. This value of $2B$ was chosen to avoid the frequency band above 0.07 cycle yr⁻¹ where the residual global temperature spectrum decreases rapidly, as shown below. Using 12 Slepian sequences, we obtain $a_0 = -0.106 \pm 0.030$ °C and $a_1 = 0.00554 \pm 0.00096$ °C yr⁻¹.

For comparison, estimates from ordinary least-squares analysis agree with the multiple-window estimates to three significant figures but, because of the lower values of the spectrum at higher frequencies, underestimate their standard deviations by a factor of five.

To assess the significance of this estimated global temperature slope, we note that Milankovitch theory predicts that at present the Earth should be cooling by ~ 0.0004 °C yr⁻¹ (refs 34, 35). Thus, solar variability aside, the null hypothesis is that the temperature trend should be slightly negative. To test this hypothesis we use a t statistic $t = [0.00554 - (-4 \times 10^{-4})] / 0.000961 = 6.18$ which, as 12 windows were used and two parameters were estimated, is characterized by approximately 10 degrees of freedom. Therefore, given that the low-frequency spectrum is approximately white (see Fig. 2b), the slope is greater than the Milankovitch prediction with probability 99.995% (ref. 36). Using a narrower bandwidth leads to fewer Slepian sequences with resistance to spectral leakage, fewer degrees of freedom and an underestimate of the slope significance. A wider bandwidth results in an invalid t statistic, as the residual spectrum decreases rapidly at frequencies > 0.07 cycle yr⁻¹ and so violates the 'locally white' assumption. Neither a jackknife variance estimate³⁷ (a non-parametric statistic sensitive to both non-gaussianity and non-stationarity, determined from the set of spectrum estimates computed with each of the windows deleted in turn), nor the stationarity test of ref. 38 show the residual series to be non-stationary. High-resolution quadratic inverse spectrum estimates provide some evidence for a ripple on the spectrum at frequencies < 0.07 cycle yr⁻¹, consistent with a 'recurrence time' in the temperature data of ~ 28 yr, but the amplitude of this ripple is not enough to change the significance of the slope.

Finally, it has been argued that the negative trend in the temperature record between 1940 and 1970 invalidates the conclusion that the temperature is increasing over the long term. To test this, we repeated the trend calculations for overlapping 30-year subsections of the Hansen-Lebedeff series. The estimated trends for each interval are shown as the short straight lines through the temperature record in Fig. 1, and the 95%-confidence region of each is bounded by the hyperbolic arcs³⁹. The line associated with the linear trend of the entire temperature record remains in the corridor collectively outlined by the subsection error bounds.

Spectrum estimates

We estimate the spectrum of the residual series $\{e(t)\}_{t=0}^{N-1}$ by subtracting the periodic components (detected by a statistical F -test²⁸) from the detrended data, and by making an adaptively weighted multiple-window estimate of the power spectral density $S(f)$ of the residuals²⁸. No frequency components above 2.5 cycle yr⁻¹ are shown to avoid artefacts from the unequal lengths of the months.

Figure 2a is the low-frequency part of the spectrum estimate of the CO₂ residuals using $2B = 0.39$ cycle yr⁻¹ and 11 windows. The spectrum is not white, so estimates using ordinary least-squares analysis of the CO₂ trend error bounds are invalid. The variance of the estimate has been calculated by jackknifing over windows^{37,40,41}, and the resulting 5% and 95% confidence limits are shown. These error bounds are consistent with the

χ^2 -distributed estimate (with 22 degrees of freedom) expected from stationary gaussian data. Because of the shortness of this series, it is unlikely that details in the low-frequency end of the spectrum have been resolved; the plotted spectrum is a compromise between frequency resolution and statistical significance.

Figure 2b shows an estimated spectrum of the Hansen-Lebedeff global average temperature residual series. To allow resolution of more details in the spectrum, a bandwidth of $2B = 0.11$ cycle yr^{-1} was used, resulting in 11 Slepian sequences

($2BT = 108 \times 0.11 = 11.88$). As noted in ref. 21, this spectrum exhibits substantial power at periods near integer multiples of the annual cycle. Because the F -test shows that this power is not a simple periodic signal in the data (recall that the Hansen-Lebedeff referencing procedure has already subtracted the annual cycle) we examine it further below.

Relations between the series

That both the CO_2 and global temperature data have positive slopes does not prove that the two series are related: As the discussion on spurious correlation in ref. 42 makes clear, the presence of the trends in each series will cause simple time-domain correlations between the two series to be high. If it is found, however, that the fluctuations of the two detrended series are coherent over a band of frequencies, then it is more likely that the two series are related.

The frequency-domain analogue of correlation, coherence⁴³⁻⁴⁵, has been applied to meteorological data for many years⁴⁶. Conventional estimates of coherence between single sections of two records are the smoothed (by a moving average) complex product of the discrete Fourier transforms of the two series, and so can be badly biased if the phase changes over the averaging band. Section-averaging methods are inappropriate for short series; not only does dividing these series into subsections result in poor frequency resolution, but the correlation between the subsections produces unreliable coherence estimates. The multiple-window approach described in refs 28 and 41 provides a less biased estimate of coherence $C(f)$ which is suitable for short series, allowing the extraction of more information from the same data.

Figure 3a shows the multiple-window magnitude-squared coherence between the Keeling CO_2 and global temperature residuals from 1958 to 1988 (produced using the same bandwidth and number of windows as the spectrum estimate of the CO_2 residuals). It is remarkable that the two series have a coherence above the 90% confidence level at frequencies < 0.6 cycle yr^{-1} with coherence exceeding 98% over much of this low-frequency band. Because multiple-window coherence estimates spaced $2B$ apart are essentially independent, the probability of observing such levels across a wide band by chance from independent series is very low, $\sim 2 \times 10^{-6}$. Thus, not only are the trend components (at frequencies ~ 0 cycle yr^{-1}) of both time series increasing, but the residuals of the two series are also coherent with high confidence in the low-frequency band.

Figure 3b is a plot of the phase of the multiple-window coherence between the two residual (trend-subtracted) series. The phase of the coherence at 0 cycle yr^{-1} is zero and, because both trends are positive, is independent of whether trends are included or not. Between 0 and 0.8 cycle yr^{-1} the phase is roughly linear, corresponding to the CO_2 series lagging the temperature series by ~ 5 months (calculated by taking the slope of this linear section of the phase⁴⁴, $\sim 120^\circ / (360^\circ \times 0.8 \text{ cycle } \text{yr}^{-1})$) in agreement with arguments that natural positive feedback mechanisms in the carbon cycle causes carbon dioxide to lag temperature in some frequency bands¹⁹ (R. Marston, personal communication). Current knowledge of these complicated interactions involving solar forcing, the Southern Oscillation and exchange of CO_2 with the oceans on various timescales, is summarized in section 6.6 of ref. 19. Also, in agreement with Keeling's hypothesis that ocean processes are dominant, we find that the coherence between the CO_2 and the Southern Hemisphere average temperature records is slightly higher than that for the Northern Hemisphere and that the observed delay of ~ 5 months between the global temperature and CO_2 is also seen in the Southern Hemisphere phase. As the Northern Hemisphere average temperature leads CO_2 by ~ 3 months, part of the ~ 5 -month delay must be due to the transport time from the Southern Hemisphere to Mauna Loa.

The hole in the phase curve near 1 cycle yr^{-1} occurs because the temperature spectrum there is dominated by a different

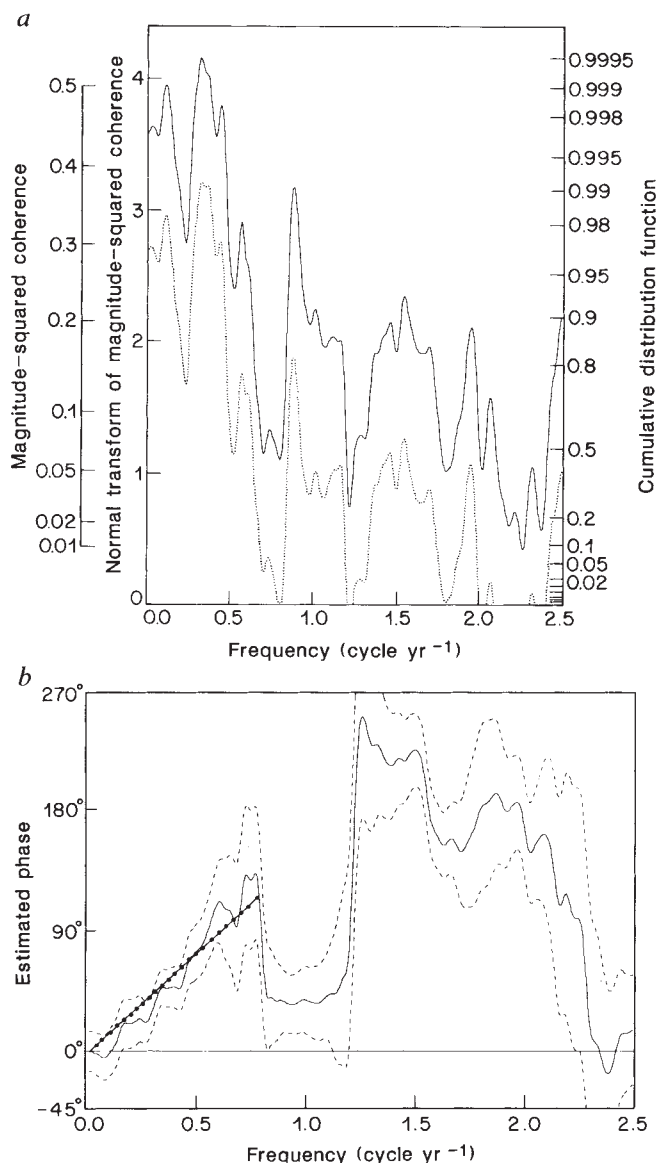


FIG. 3 a, Magnitude-squared coherence (MSC), between the Keeling CO_2 series and the global temperature series during 1958-1988. The vertical axis on the far left is the value of the MSC, and the axis on the left of the graph is the value of the coherence magnitude transformed by $\tanh^{-1}$. On this scale the coherence estimates should be roughly gaussian with unit standard deviation⁴⁹. The vertical axis on the right gives the cumulative distribution function for incoherent series. The upper (solid) curve represents the transformed coherence values $\{\tanh^{-1}(|C(f)|)\}$, and the lower (dashed) curve is offset by one standard deviation as determined by jackknifing over windows. b, Phase of coherence between the Keeling CO_2 series and the global temperature series during 1958-1988 corresponding to the MSC in a. The two dashed lines show the ± 1 s.d. limits obtained by jackknifing. The low-frequency trend in the phase corresponds to a delay of ~ 5 months, whereas the 'hole' near 1 cycle yr^{-1} reflects the predominance of other effects near this frequency. The poorer limits on the phase at higher frequencies is a consequence of the lower coherences there. These jackknife estimates agree with gaussian theory.

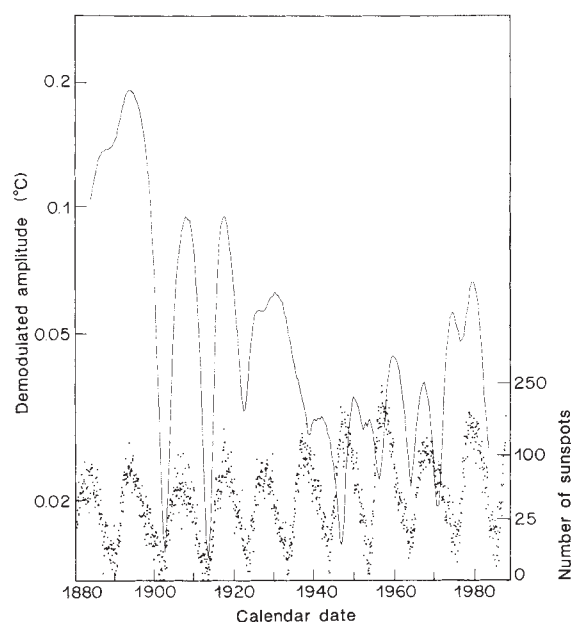


FIG. 4 The solid curve shows the amplitude of the complex demodulate of the Northern Hemisphere temperature record between 0.9 and 1.1 cycle yr^{-1} . A plot of the sunspot numbers is shown below. Detailed examination of the coherence between the demodulate and the solar cycle shows that these two have about a $1/(30 \text{ yr})$ difference in frequency (corresponding to the stronger lower sideband mentioned in Fig. 2b).

physical mechanism, and because of their different phase characteristics, we analyse the Northern and Southern Hemisphere temperature records separately in this frequency band. To do this, a multiple-window bandpass-filtered temperature series is formed by expanding the residual temperature series on a set of Slepian sequences that have most of their spectral energy confined between 0.9 and 1.1 cycle yr^{-1} . The resulting 'complex demodulate' for the Hansen-Lebedeff Northern Hemisphere temperatures is shown by the solid line in Fig. 4. The sunspot record (solid dots in Fig. 4) is clearly similar to the demodulated temperature data, suggesting that the annual signal is modulated by fluctuations in solar output. Detailed analysis shows that coherence between the sunspot and filtered temperature series is significant, and similar to the non-stationary structure found

in ref. 47. The phase of the sunspot-filtered temperature coherence has a linear drift, corresponding to a frequency difference between these two oscillations of $1/\Delta$, where $\Delta = 27\text{--}30 \text{ yr}$, possibly related to the low-frequency ripple in the temperature spectrum mentioned above. The results for the Southern Hemisphere are similar.

Discussion

The coherence results presented here provide significant evidence that the average global temperature and CO_2 concentration from 1958 to 1988 are linearly related at many frequencies. But caution must be exercised in interpreting this result as suggesting that the variations in atmospheric CO_2 are causing the changes in global temperature, even though there are plausible physical mechanisms linking the two series. Apparent correlations that are used to postulate causality can sometimes be misleading, as in the case involving timing of earthquakes⁴⁸. In addition, one should be particularly cautious in interpreting the coherence when the series analysed are as short as these; climatic and solar variations often are of longer duration than these records.

From atmospheric chemistry, global temperature depends nonlinearly on CO_2 concentration (T. Graedel, personal communication). The procedure used here implicitly uses a linear dependence. Bispectral estimates provide evidence of quadratic terms, although the shortness of these series makes this difficult to quantify. Also, except for the solar modulation of the temperature series near 1 cycle yr^{-1} , we have ignored the cyclostationary properties of these two series (that is, the statistics of these series vary periodically).

A more complete analysis would include estimates of the coherences between the various global average temperature time series, records of atmospheric CO_2 concentration, human CO_2 production, sunspots, volcanic activity and the Southern Oscillation Index, which are all high in various frequency bands and have complicated phase interrelations. For example, the coherence between the detrended Keeling CO_2 series and the Southern Oscillation Index is high near 0.4 and 2.4 cycle yr^{-1} . If we calculate the coherence between the CO_2 and global temperature series from which terms describing their linear dependences on the Southern Oscillation Index and sunspot record have been subtracted (called a partial coherence), the low-frequency magnitude-squared coherence increases to almost 0.7 whereas it decreases near 0.3 cycle yr^{-1} . Several of these series also exhibit an oscillation at an apparent period of ~ 15 years. Further analysis of their multivariate relations will be described elsewhere in more detail. □

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Self-reactive $\gamma\delta$ T cells are eliminated in the thymus

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The genes encoding a $\gamma\delta$ T-cell receptor specific for a major histocompatibility complex class I molecule encoded by the Tla locus have been inserted into the mouse germ line. In mice that do not express the Tla-encoded determinant, transgenic $\gamma\delta$ T cells are a functional component of the CD4⁺CD8⁺ 'double-negative' T cells in the thymus and peripheral lymphoid organs. In mice that express the Tla-encoded determinant, there are no transgenic $\gamma\delta$ T cells in peripheral lymphoid organs, and there are no thymocytes expressing normal levels of the transgenic $\gamma\delta$ T-cell receptor.

EXPRESSION of $\gamma\delta$ T-cell antigen receptors (TCR) defines a distinct T-cell subset in lymphoid organs such as thymus, spleen and lymph nodes¹⁻⁸. Murine $\gamma\delta$ T cells differ in two characteristic respects from T cells bearing $\alpha\beta$ TCR. First, $\gamma\delta$ T cells do not usually express the T-cell accessory molecules CD4 and CD8^{9,10}, which are expressed in a mutually exclusive pattern on virtually all peripheral $\alpha\beta$ T cells¹¹. Second, $\gamma\delta$ T cells are often the predominant T-cell subset in tissues not considered to be classical lymphoid organs, including the epithelium of the skin¹², intestine¹³ and lung¹⁴.

One of the important issues concerning $\gamma\delta$ T cells is whether they develop in the thymus in a manner similar to $\alpha\beta$ T cells¹⁵. The development of $\alpha\beta$ T cells in the thymus involves two main types of selection: positive selection, which selects the cells bearing TCRs recognizing the particular allele of a major histocompatibility complex (MHC) molecule expressed on the thymus epithelium, and negative selection, which deletes cells bearing overtly self-reactive TCRs (for reviews, see refs 16 and 17). An important question is whether these types of selection apply to $\gamma\delta$ T-cell development. To investigate $\gamma\delta$ T-cell development, we have constructed transgenic mice containing productively rearranged γ - and δ -TCR genes from an alloreactive $\gamma\delta$ T-cell clone, G8 (ref. 18). These transgenic mice have allowed us to study directly the principles of tolerance and selection that govern $\gamma\delta$ T-cell development in the thymus.

The $\gamma\delta$ T-cell clone G8, derived from BALB/c (H-2^d) *nu/nu* mice, is specific for an allelic MHC class I gene product encoded in the Tla region of the MHC¹⁹. The G8 clone does not react with antigen-presenting cells (APCs) from 'self' H-2^d mice, but reacts with APCs from mice of several other MHC haplotypes, including H-2^k and H-2^b. The G8 clone has phenotypic characteristics typical of $\gamma\delta$ T cells in that it does not express CD4 or

CD8 cell-surface molecules¹⁸. The γ -chain of G8 is composed of V γ 2-J γ 1-C γ 1 elements¹⁸, the most common γ -chain isotype expressed by adult thymus $\gamma\delta$ T cells^{9,15}. A monoclonal antibody has been generated with a specificity limited to $\gamma\delta$ TCRs containing the V γ 2 segment (S.M.W., L.A.M. and J.A.B., manuscript in preparation). This antibody reacts with very few cells from adult spleen or thymus, and is therefore functionally specific for cells expressing the transgenic $\gamma\delta$ TCR. The δ -chain of G8 is composed of a previously undescribed member of the V α 11 family, which is rearranged to J δ 1 (ref. 18).

Tolerance of $\gamma\delta$ T cells in the thymus

We used genomic clones containing the G8 γ - and δ -TCR genes (Fig. 1) to generate (C57BL/6J $\times$ BALB/c) F₂ transgenic mice²⁰. We obtained seven lines of transgenic mice. Initially, we analysed the offspring of a male transgenic founder no. 75. We estimate that this founder had four copies of both the γ - and δ -chain transgenes co-integrated into its genome, and typed it as MHC H-2^{b/d}. We bred the founder to a BALB/c female to generate transgenic offspring that were H-2^{b/d} and H-2^{d/d}.

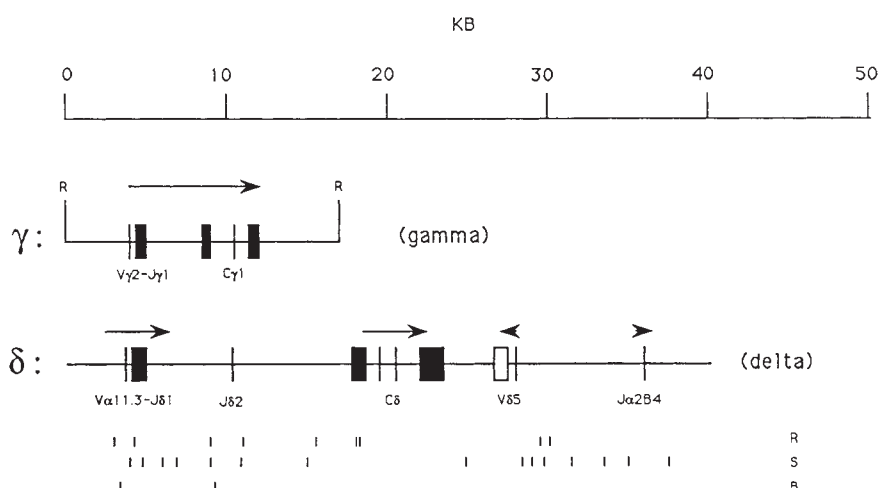
Thymuses were collected from 5-week-old transgenic and non-transgenic littermates of the cross described above and analysed them for expression of CD3, V γ 2 and $\alpha\beta$ TCR by flow cytometry (Fig. 2). Non-transgenic thymuses showed typical levels of CD3 and $\alpha\beta$ TCR expression²¹: ~15% of cells stained CD3-bright/ $\alpha\beta$ TCR-bright, corresponding to mature T cells, and a large fraction of cells stained CD3-low/ $\alpha\beta$ TCR-low, corresponding to immature thymocytes. There was no detectable V γ 2 staining in non-transgenic thymuses.

H-2^{d/d} transgenic thymuses could be characterized by two main phenotypes (type 1 and type 2, Fig. 2). In type-1 thymuses, ~55% of the cells stained brightly with the anti-V γ 2 antibody, which correlated with the number of CD3-bright cells (Fig. 2). The number of $\alpha\beta$ TCR-bright cells was diminished in type-1 thymuses (2% of total cells, Fig. 2). By contrast, the pattern of $\alpha\beta$ TCR expression in type-2 H-2^{d/d} transgenic thymuses was similar to the nontransgenic pattern, and contained a smaller fraction of V γ 2-staining cells (17%) than did the type-1 thymuses. Two-colour staining of V γ 2 versus $\alpha\beta$ TCR revealed no double-staining cells (data not shown); therefore V γ 2- and $\alpha\beta$ TCR-expressing cells belong to two separate cell populations in type-2 thymuses. The variation in the H-2^{d/d} transgenic mice does not seem to be dependent on the age of the mice or the number of thymocytes. All of the H-2^{d/d} transgenic thymuses examined were smaller than nontransgenic thymuses, each containing 14×10^6 cells on average, compared with an average of 100×10^6 cells per normal thymus.

We analysed the effect of a self-reactive $\gamma\delta$ TCR on T-cell development in H-2^{b/d} transgenic littermates. H-2^{b/d} transgenic

FIG. 1 TCR gene constructs used to make transgenic mice. Both constructs are unaltered genomic clones isolated from two different libraries made with genomic DNA from the $\gamma\delta$ T cell G8. The γ -chain gene construct is a 17-kilobase (kb) λ phage clone that is identical, except for the V-J junction, to the $V\gamma 2$ - $J\gamma 1$ - $C\gamma 1$ clone described in ref. 34. The δ -chain gene construct is a cosmid clone containing a $V\alpha 11$ -like V gene rearranged to $J\delta 1$. The positions of $V\delta 5$ and $J\alpha 2B4$ are inferred from ref. 35. Both clones contain productively rearranged variable regions, as determined by subcloning and sequencing (data not shown). The sequence of the junction of the γ -chain gene from the last cysteine codon of $V\gamma 2$ to the first glycine codon of $J\gamma 1$ is TGTTCTACGGGGAGGGGAGCTCAGGT. The sequence of the junction of the δ -chain gene has been published¹⁸. Restriction-enzyme sites: B, *Bam*HI; R, *Eco*RI; S, *Sst*I. Arrows indicate direction of transcription.

METHODS. Genomic DNA was prepared from the T-cell clone G8 described in ref. 37. The DNA was completely digested with *Eco*RI and ligated to *Eco*RI-digested λ DASH arms (Stratagene) according to the manufacturer's specifications. After packaging using Gigapack Plus (Stratagene), the resulting library was screened with a $V\gamma 2$ -specific probe using standard procedures³⁷, and several identical γ -clones were obtained. The 17-kb γ -insert was subcloned into the plasmid pKS (Stratagene) using *Eco*RI. The cosmid library was constructed essentially as described³⁸, using the cosmid vector scos. The library was screened with a $J\delta 1$ -specific probe³⁹,



and positive clones were rescreened with a $V\alpha 11$ -specific probe³⁶. Overlapping δ -gene clones were characterized and mapped according to Evans *et al.*³⁸, the δ -gene clone used was chosen by virtue of a long region downstream of $C\delta$. TCR γ - and δ -gene clones were completely digested with *Eco*RI or *Not*I, respectively, to remove vector sequences, and inserts were purified for transgenic injection over 10–60% sucrose gradients. The γ - and δ -gene inserts were injected together at a 1:1 ratio into the male pronuclei of fertilized eggs using standard procedures²⁰.

Founder no. 75

Thymus

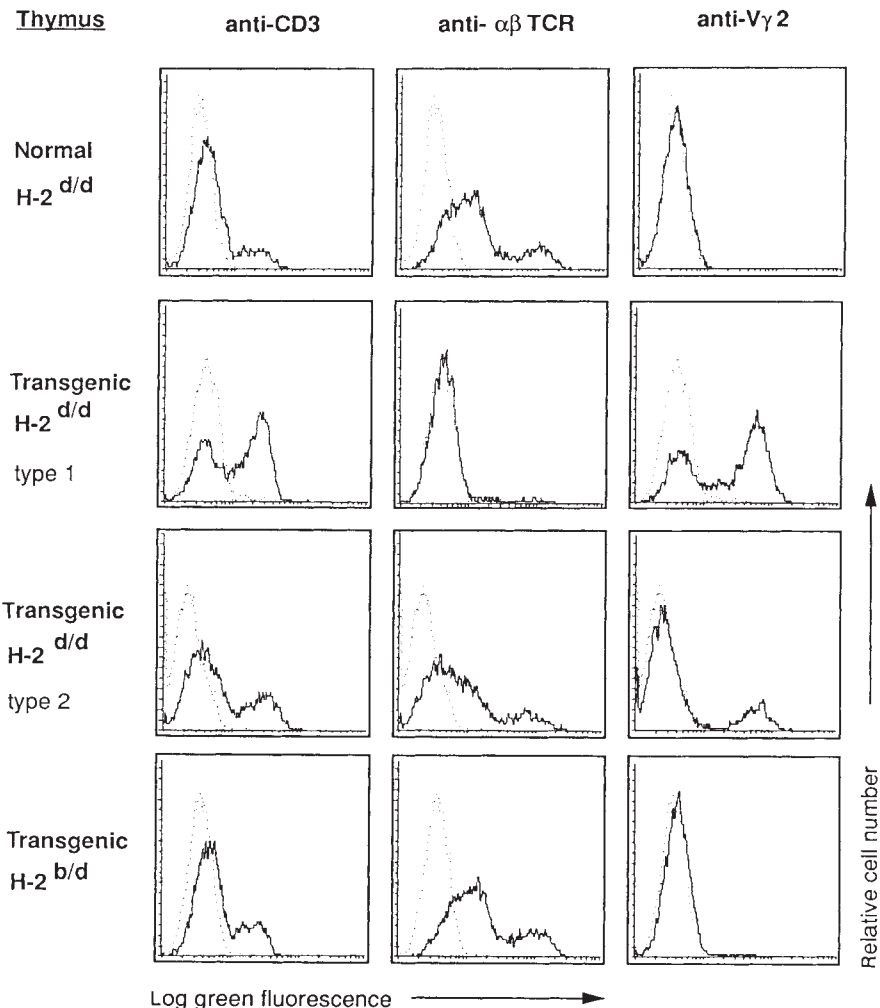


FIG. 2 TCR expression in the thymuses of non-transgenic, non-self-reactive transgenic, and self-reactive transgenic littermates.

METHODS. Thymuses were removed from 5-week-old offspring mice from the cross (founder no. 75 $\times$ BALB/c), and single-cell suspensions made. Presence of the transgenes was detected by Southern-blot analysis of tail DNA²⁰ using $V\gamma 2$ and $J\delta 1$ -specific probes. Staining was performed on cells washed once in Hank's balanced salt solution and once in staining buffer (PBS, 2% FCS and 0.1% sodium azide). Anti-CD3 (145-2C11)⁴⁰ and anti- $V\gamma 2$ (UC3-10A6; S.M.W., L.A.M. and J.A.B., manuscript in preparation) antibodies were tissue-culture supernatants used neat. Anti- $\alpha\beta$ (H57-597)²¹ was ascites used at 10^{-5} dilution. Cells 10^6 were stained in 100 μ l antibody or staining buffer (secondary control) for 1 h at 4 $^{\circ}$ C, washed once in staining buffer, then resuspended with 50 μ l fluorescein isothiocyanate (FITC) coupled goat anti-hamster immunoglobulin (Caltag, South San Francisco) for 145-2C11, H57-597 and UC3-10A6 for 30 min at 4 $^{\circ}$ C. Secondary reagents were used at a final concentration of 1:50. The cells were then washed twice in staining buffer, and resuspended in 500 μ l staining buffer with 1% formaldehyde. Five thousand cells per sample were analysed on a FACS flow cytometer (Becton Dickinson using FACScan Research Software. Dead cells were gated out of analysis by virtue of forward and sideways light-scatter patterns. H-2 haplotypes were determined by staining with the anti H-2 K^b antibody Y-3 (ref. 41), using a goat anti-mouse secondary reagent (Caltag).

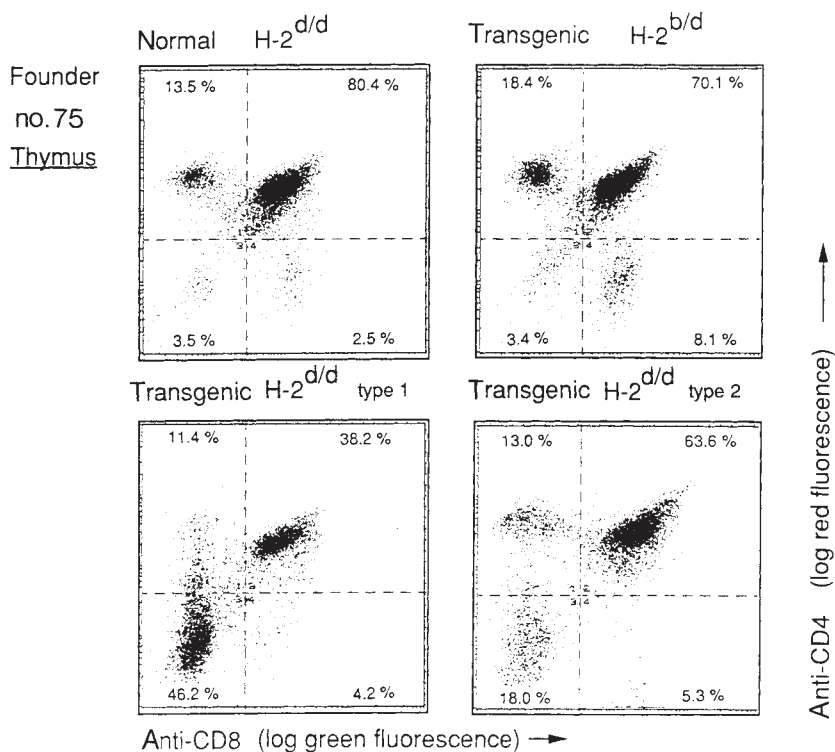


FIG. 3 CD4 and CD8 expression in the thymuses of nontransgenic, non-self-reactive transgenic, and self-reactive transgenic littermates. METHODS. CD4 and CD8 staining populations were determined by co-staining with phycoerythrin E (PE)-conjugated anti-CD4 and FITC-conjugated anti-CD8 (Becton Dickinson). CD4-CD8 quadrants were set using normal thymus as a standard.

thymuses contained normal numbers of cells. In transgenic $H-2^{b/d}$ mice, the transgenic $\gamma\delta$ TCR, CD3 and $\alpha\beta$ TCR staining patterns were almost identical to the nontransgenic patterns, including the virtual absence of $V\gamma 2$ -staining cells (Fig. 2). These thymuses contained a small population of cells (1–2% of total), not found in normal mice, that stained weakly with anti- $V\gamma 2$ antibody.

Transgenic TCR expressed on $CD4^-CD8^-$ cells

The T-cell markers CD4 and CD8 delineate four stages of T-cell development in the thymus (for reviews, see refs 16 and 17). $CD4^+CD8^-$ and $CD4^-CD8^+$ 'single-positive' thymocytes generally represent mature $\alpha\beta$ T cells that have been both positively

and negatively selected, whereas $CD4^+CD8^+$ 'double-positive' (DP) thymocytes are precursors of mature T cells. $CD4^-CD8^-$ 'double-negative' (DN) thymocytes comprise ~5% of thymocytes, and this phenotype includes $CD3^-$ early precursor T cells^{22,23}, $\gamma\delta$ T cells^{1,2} and a small population of $\alpha\beta$ T cells that predominantly express $V\beta 8$ (refs 24, 25).

The analysis of a thymus from a nontransgenic animal is shown in Fig. 3; the pattern shown is constant among normal mice. In the type-1 $H-2^{d/d}$ transgenic thymus (Fig. 3) there was a decrease in the number of $CD4^+CD8^+$ cells (38% of total cells), and a concordant increase in $CD4^-CD8^-$ cells to 46%. The percentage of $CD4^+CD8^-$ and $CD4^-CD8^+$ cells were not altered, although there was an apparent decrease in CD4-bright

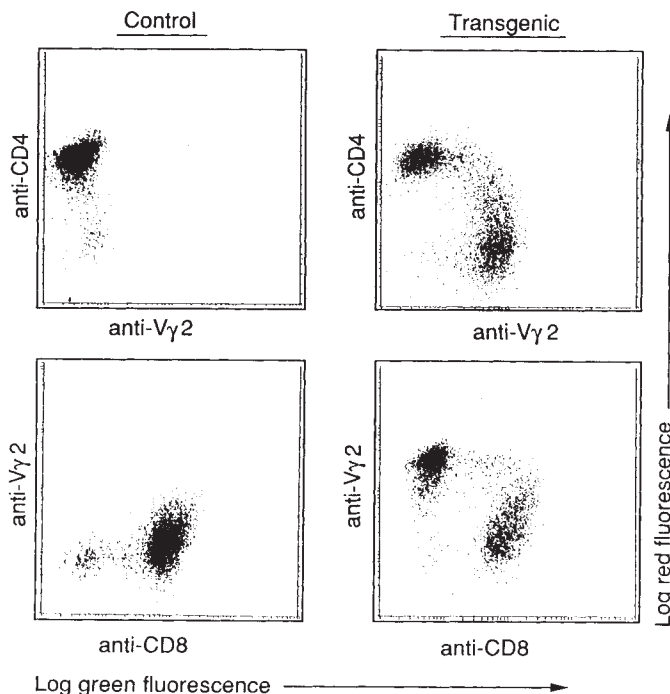


FIG. 4 $V\gamma 2$ expression versus CD4 and CD8 expression in $H-2^{d/d}$ transgenic and nontransgenic thymus. $CD4^+V\gamma 2^+$ cells constituted 16% of total cells; $CD8^+V\gamma 2^+$ cells constituted 12% of total cells in the transgenic thymus. Because there are only 4% CD8 single-positive cells in this thymus, the remaining 8% $V\gamma 2^+CD8^+$ cells had to be contained in the double-positive population. As there were 40% double-positive cells in this thymus, 8% $V\gamma 2^+CD8^+$ corresponds to ~20% of the total double-positive population. METHODS. Two-colour staining was performed with the CD4 and CD8 reagents described in Fig. 3, and the anti- $V\gamma 2$ reagent in Fig. 2. Cells were stained first with anti- $V\gamma 2$, washed, then stained with anti-CD4 or anti-CD8 antibody, washed, then stained with goat anti-hamster immunoglobulin. For two-colour staining with anti-CD4 antibody, FITC-coupled goat anti-hamster immunoglobulin (Fig. 2) was used as a secondary stain. For two-colour staining with anti-CD8 antibody, biotinylated goat anti-hamster immunoglobulin (Vector Labs Inc., Burlingame, California) was used as a secondary stain, followed by incubation (30 min, 4 °C) with PE-coupled avidin D (Vector Labs).

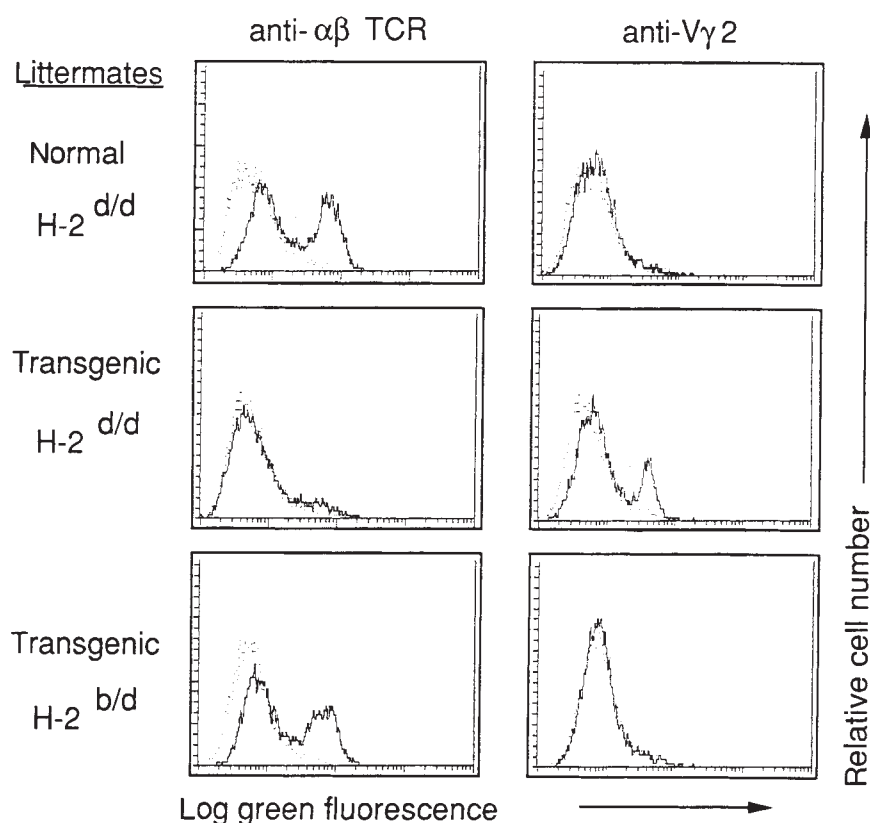


FIG. 5 TCR expression in the spleen of normal and transgenic littermates. The $\alpha\beta$ T cells constituted 33 and 35% of cells in normal and $H-2^{b/d}$ transgenic spleen, respectively, whereas $\alpha\beta$ T cells constituted 8% of cells in $H-2^{d/d}$ transgenic spleen. The percentage of CD4-staining cells plus the percentage of CD8-staining cells (data not shown) was equal to the percentage of $\alpha\beta$ -staining cells in all three cases. The total number of T cells equalled the number of $\alpha\beta$ T cells in normal and $H-2^{b/d}$ transgenic spleen, whereas the total number of T cells in the $H-2^{d/d}$ transgenic spleen equalled 25% ($V\gamma 2^+$ cells and $\alpha\beta$ TCR cells). METHODS. Spleens were removed from 5-week-old offspring mice from the cross (founder no. 75 $\times$ BALB/c), single-cell suspensions made, and red blood cells lysed by hypotonic shock. Staining was performed as in the legend to Fig. 2. Spleen-cell yields from both types of transgenic littermates were 50–60% of the yield from normal mice (10^8 cells).

single-positive cells; most $CD4^+CD8^-$ cells seemed to express low levels of CD4. The type-2 transgenic thymus also contained an increased percentage of $CD4^+CD8^-$ cells, consistent with the number of $V\gamma 2$ -staining cells (18%) and a concordant decrease in $CD4^+CD8^+$ DP cells (Fig. 3). The percentages of $CD4^+CD8^-$ and $CD4^+CD8^+$ cells in the type-2 thymus were not different from normal.

Two-colour staining of $V\gamma 2$ versus CD4 and $V\gamma 2$ versus CD8 (Fig. 4) revealed that 55–70% of $\gamma\delta$ T cells in the type-1 transgenic thymus were in the population of $CD4^+CD8^-$ cells, which is the same phenotype as the G8 $\gamma\delta$ T-cell clone. The transgenic $\gamma\delta$ TCR therefore seems to determine a predominant $CD4^+CD8^-$ phenotype for the developing $\gamma\delta$ T cell. At least 20% of DP cells were $V\gamma 2$ -positive in this $H-2^{d/d}$ transgenic thymus (see Fig. 4, legend). These numbers are consistent with those of $CD4^+CD8^+$ DP $\gamma\delta$ T cells previously observed in adult thymus²⁶. DP $\gamma\delta$ T cells could represent either immature cells destined to become $CD4^+CD8^-$, or precursors of $\gamma\delta$ T cells that express CD4 and CD8 in the spleen and epithelia (refs 8, 26; S.M.W. and J.A.B., unpublished results).

The $CD4$ - $CD8$ expression pattern of a transgenic $H-2^{b/d}$ thymus indicated a preponderance of $CD4^+CD8^+$ cells, and only a small percentage of $CD4^+CD8^-$ cells (Fig. 3). This result is in agreement with the relatively normal CD3 and $\alpha\beta$ TCR phenotype of this type of thymus. The transgenic $H-2^{b/d}$ thymuses had reproducibly fewer $CD4^+CD8^+$ cells than did thymuses from nontransgenic mice. This decrease in $CD4^+CD8^+$ cells was reflected in an increase in both CD4 and CD8 single-positive cells. Increased numbers of CD3-bright and $\alpha\beta$ TCR-bright cells were also seen in the transgenic $H-2^{b/d}$ thymus (Fig. 2; 23% versus 15% for a nontransgenic thymus), whereas the number of $CD4^+CD8^-$ cells was unchanged.

Expression in peripheral lymphoid organs

We next determined whether the $\gamma\delta$ thymocytes could migrate from the thymus to populate the peripheral lymphoid organs. We analysed splenocytes from nontransgenic, $H-2^{d/d}$ transgenic,

and $H-2^{b/d}$ transgenic littermates by flow cytometry (Fig. 5). The nontransgenic and transgenic $H-2^{b/d}$ spleens had similar percentages of $\alpha\beta$ TCR-staining cells (33% and 35%, respectively). Both types of spleen had the same proportion of $CD4^+$ and $CD8^+$ cells (data not shown), and we found no detectable $V\gamma 2$ -staining cells in either type of spleen. By contrast, the type-1 $H-2^{d/d}$ transgenic spleen contained only 8% $\alpha\beta$ T cells, whereas 16% of the cells were $V\gamma 2$ -positive. Two-colour staining (data not shown) shows that most (at least 80%) peripheral $V\gamma 2$ -bearing cells were $CD4^-$ and $CD8^-$. The ratio of $\gamma\delta$ T cells to $\alpha\beta$ T cells in the type-1 $H-2^{d/d}$ transgenic spleen (2:1) is much lower than in the thymus (about 28:1). A lower ratio of $\gamma\delta$ T cells to $\alpha\beta$ T cells in the spleen than in the thymus has also been observed with type-2 $H-2^{d/d}$ transgenic mice, although the shift is not as dramatic, owing to a larger population of $\alpha\beta$ T cells in the thymus of these mice.

To test for both peripheral function and transgene specificity, we measured the proliferative response of the splenocytes from transgenic mice in a mixed lymphocyte reaction. The 5-week-old type-1 $H-2^{d/d}$ transgenic spleen produced a strong response to $H-2^b$ (B10) APCs, and a fivefold weaker response to B10.BR ($H-2^k$) APCs (Fig. 6). The $H-2^{d/d}$ transgenic splenocytes did not respond to self (BALB/c)-APCs or to B10.A ($H-2^k$, I^k and D^d) APCs; this pattern of reactivity has been previously shown for the clone G8 (ref. 18). Cell lines grown from similar cultures showed an identical allo-MHC response, expressed $V\gamma 2$ at the cell surface, and produced $V\alpha 11$ messenger RNA, as detected by northern-blot analysis (data not shown). These results indicate that $\gamma\delta$ transgenes transferred the specificity of the G8 $\gamma\delta$ clone. The use of the transgenic δ -chain gene was demonstrated by analysing $H-2^{d/d}$ transgenic thymus DNA for $J\delta 1$ rearrangements (data not shown). Only the germ-line and transgenic $J\delta 1$ bands were detectable, indicating the lack of endogenous δ -chain gene rearrangements in these $\gamma\delta$ T cells. Consistent with the deletion of $\gamma\delta$ T cells and maintenance of self-tolerance, the spleen of the $H-2^{b/d}$ transgenic did not respond at all to $H-2^b$ or $H-2^d$ APCs, but responded weakly to

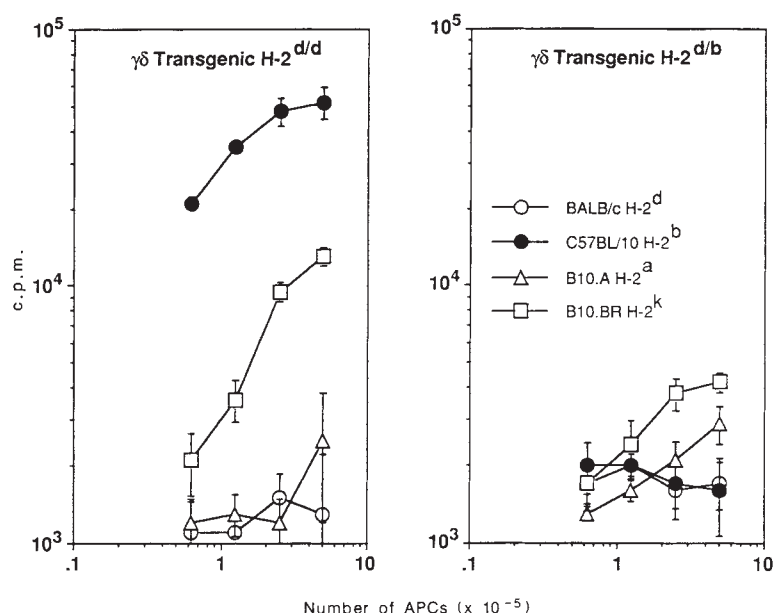


FIG. 6 Functional activity of the $\gamma\delta$ TCR transgenes in the spleen of H-2^{b/d} versus H-2^{d/d} transgenic mice as measured by proliferation in a mixed lymphocyte reaction.

METHODS. Normal or transgenic spleen cells 2×10^5 were incubated with 5×10^5 irradiated (3,500 rad) APC per well in a 96-well microtitre plate. The cultures were pulsed after 72 h with ^3H -labelled thymidine, collected after 96 h, and ^3H -labelled thymidine incorporation measured by liquid scintillation counting. APCs were spleen cells from the different mouse strains indicated.

B10.BR (H-2^k) and B10.A (H-2^a) APCs (Fig. 6). The response was at least as high as that of a nontransgenic littermate (data not shown), indicating the deletion of self-reactive $\gamma\delta$ T cells does not affect the ability of the animal to generate an allogeneic response mediated by $\alpha\beta$ T cells.

Discussion

Maintenance of self-tolerance by clonal deletion. We used transgenic mice carrying an alloreactive $\gamma\delta$ TCR to demonstrate that the immune system can impose tolerance in $\gamma\delta$ T cells in a manner that seems to be related to that shown for $\alpha\beta$ T cells^{27,28}. We found that in an H-2^d thymus, not expressing a ligand recognized by the $\gamma\delta$ TCR, thymocytes bearing transgenic $\gamma\delta$ TCR constituted a large fraction of the population. In an H-2^b thymus, in which the transgenic $\gamma\delta$ T cells would be self-reactive, this cell population was virtually eliminated. In this latter situation, there was a detectable number of V γ 2 low-staining cells (<2%), and almost normal numbers of $\alpha\beta$ T cells. The remaining V γ 2-bearing cells could have represented transgenic $\gamma\delta$ T cells that were actively being tolerized, cells that did not express the δ -chain, or cells that had escaped deletion by an unknown mechanism. A similar pattern of negative selection has been seen in five other founder strains. In some mice there is a decrease in the absolute number of $\alpha\beta$ T cells, and so the percentage of V γ 2 low-staining cells is higher. In all H-2^{b/b} mice examined, there were no detectable V γ 2-bearing cells in the spleen.

Phenotypic selection of $\gamma\delta$ T cells. In TCR $\alpha\beta$ transgenic mice there is selection among transgene-bearing T cells for the CD4 or CD8 phenotype, depending on the receptor specificity for MHC class II or class I molecules, respectively^{29,30}. The data presented here indicate that the presence of the $\gamma\delta$ TCR also seems to determine the CD4-CD8 phenotype. Most of the V γ 2-bearing T cells are CD4⁺CD8⁺, the same phenotype as the G8 T cell clone. If the specificity of the $\gamma\delta$ TCR does indeed determine the CD4-CD8 phenotype, then these cells could undergo a selection step analogous to that for $\alpha\beta$ T cells. Alternatively, the $\gamma\delta$ TCR could be expressed exclusively in a separate thymocyte lineage regardless of receptor specificity, that is characteristically CD4⁺CD8⁺.

Relationship of $\alpha\beta$ versus $\gamma\delta$ T-cell lineage. The relationship between T cells expressing $\gamma\delta$ TCRs and those expressing $\alpha\beta$ TCRs has been extensively discussed^{17,31-33}. Simply stated, the issue is whether the thymic stem cells split into two separate

developmental lineages before gene rearrangements or whether the outcome of $\gamma\delta$ - and $\alpha\beta$ -gene rearrangements determines the cell lineage. In our transgenic mice, $\alpha\beta$ and $\gamma\delta$ T cells seem to be of two separate lineages. Clearly, in type-2 H-2^{d/d} transgenic mice, $\alpha\beta$ T cells can develop as a distinct population from transgenic $\gamma\delta$ T cells despite all the cells possessing rearranged copies of the γ - and δ -chain genes. Also, in H-2^{b/d} mice, self-reactive transgenic $\gamma\delta$ T cells can be deleted without precluding $\alpha\beta$ T-cell development; this implies that the $\alpha\beta$ T cells do not go through a precursor stage of $\gamma\delta$ TCR expression. These data indicate that thymocytes destined to express the $\alpha\beta$ receptor do not express the γ - and δ -chain genes, allowing the cells to proceed through the normal $\alpha\beta$ T-cell developmental pathway. The development of separate populations of $\alpha\beta$ and $\gamma\delta$ T cells in the thymus has also been observed with four other transgenic founders, and is thus probably independent of the copy number and position of the $\gamma\delta$ transgenes. In addition, the maximum number of transgenic $\gamma\delta$ T cells that we have observed in an H-2^{d/d} thymus is 12×10^6 , with an average of $\sim 7 \times 10^6$. This contrasts with results from $\alpha\beta$ TCR transgenic mice: the thymuses from these mice contained up to 99% transgenic $\alpha\beta$ TCR-bearing cells, representing at least 100×10^6 cells²⁹. These data imply that in transgenic mice only expressing the $\gamma\delta$ TCR, the $\alpha\beta$ T-cell population is not replaced by cells bearing a $\gamma\delta$ TCR.

In conclusion, the absence of self-reactive $\gamma\delta$ T cells in the thymus and peripheral lymphoid organs implies that clonal deletion operates to maintain tolerance in the $\gamma\delta$ T-cell lineage. The data in this report also provide evidence that $\gamma\delta$ and $\alpha\beta$ T cells belong to two separate lineages of T cells, and that expression of the $\gamma\delta$ TCR can be inhibited in the $\alpha\beta$ T-cell lineage. □

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***In vitro* replication through nucleosomes without histone displacement**

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A well-characterized set of proteins encoded by bacteriophage T4 replicates DNA *in vitro* and generates replication forks that can pass nucleosomes. The histone octamers remain associated with newly replicated DNA even in the presence of excess DNA competitor, and intact nucleosomes re-form on the two daughter DNA helices. It is concluded that nucleosomes are designed to open up transiently to allow the passage of a replication fork without histone displacement.

THE fundamental unit of eukaryotic chromatin is the nucleosome—a disc-like structure composed of two each of the four histones H₂A, H₂B, H₃ and H₄, and ~145 base pairs (bp) of DNA wrapped around the outside of this octameric histone core. The histones condense the DNA by forming nucleosomes and higher-order structures. In addition, the variability in histone structure and modification allow the formation of different types of nucleosomes and therefore different states of chromatin (for a review, see ref. 1). These different chromatin states are thought to have a role in determining the transcriptional capability of specific chromosomal regions (for a review, see ref. 2). Each time a cell divides, it is not just the DNA that must be replicated, but the entire complex structure of the chromatin. Several studies indicate that specific features of transcriptionally competent chromatin are reproduced behind the replication fork on both daughter strands^{3–5}. How this is accomplished is not known. In principle, the parental histones could be directly inherited as the replication fork passes, and these old histones could then serve as a template to help define the post-replicative distribution of the new ones^{6–9}. Indeed, there is evidence that the parental histone octamers are not disrupted by the replication process and persist in cells through several generations^{8,10,11}. But attempts to determine the mode of segregation of the parental histones to the replicated daughter strands

have given contradictory results. Some data support a conservative segregation of all of the parental histones to the daughter DNA helix made on the leading-strand of the fork, where DNA is synthesized continuously^{10,12–14}. Other data indicate, however, that the histone octamers are distributed to both of the daughter helices behind the fork^{11,15–22}. For example, Sogo *et al.*²² have used electron microscopy to study nucleosome segregation during simian virus 40 (SV40) DNA replication by blocking new histone synthesis with cycloheximide and then using psoralen crosslinking to mark the positions of the old nucleosomes inherited behind the fork *in vivo*. Under these conditions, nucleosomes were seen to be inherited in small clusters that were equally distributed on both daughter DNA helices. Because these nucleosomes were not present immediately behind the fork, it was proposed that the histone octamers dissociate transiently as the replication fork passes, but quickly reassociate with the newly replicated DNA.

Do the histones remain permanently associated with the DNA or are they transiently removed during the passing of the fork? We have now addressed this question by directly studying what happens to a nucleosome when a replication fork passes through it *in vivo*. We began by assessing the ability of the highly purified *in vitro* T4 bacteriophage DNA replication system to replicate through nucleosomes. We found that the T4 replication fork can replicate past histone octamers on DNA. This allowed us to determine the fate of these histones during the replication process with a completely defined system that contains only nine highly purified T4 replication proteins, the four nucleosomal histones and a purified DNA template.

The artificial chromatin template

The nucleosome-containing DNA template that we used for replication with the T4 *in vitro* replication system is a 4.7-kilobase (kb) circular plasmid DNA containing the replication origin of bacteriophage M13. Nicking this origin with the bacteriophage gene 2 protein provides a unique 3' end that serves as a starting site for initiation of *in vitro* DNA synthesis²³. Unfortunately, this origin region is also a preferential site for nucleosome assembly. To preserve the accessibility of the cutting site of the gene 2 protein, we restricted nucleosome assembly on this template to only a few nucleosomes per DNA molecule. We added nucleosomes to more or less random positions on

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the template by transferring ^3H -lysine-labelled histone octamers from a nucleosome donor (the unusually stable nucleosome reconstituted on a DNA fragment containing a 5S ribosomal RNA gene)²⁴ to the circular supercoiled form of the template DNA molecule.

The reconstituted nucleosomes seem to be authentic in that they contain a full set of histones (Fig. 1a), with the same relative composition of the four histones as the original donor nucleosomes. Also, digestion of the nucleosome-containing DNA templates with micrococcal nuclease produces a DNA fragment of ~ 145 bp, similar in size to the DNA fragment released from native nucleosome cores (Fig. 1b). The specific activity of the reconstituted DNA-histone complexes ($\sim 13,000$ c.p.m. μg^{-1}) indicates the transfer of about three histone octamers per DNA template. This value is in agreement with the number of nucleosomes observed by electron microscopy for most of the DNA templates (Fig. 1c). But we found some template molecules with no nucleosomes, whereas others contained up to six or seven nucleosomes.

The fork can pass nucleosomes after a pause

Seven bacteriophage T4-encoded proteins reconstitute an *in vitro* DNA replication system that catalyses coupled leading- and lagging-strand DNA synthesis at a replication fork²⁵. The proteins involved are the T4 DNA polymerase holoenzyme (the products of T4 genes 43, 44/62 and 45), a helix-destabilizing

single-stranded binding (SSB) protein (gene 32 protein) and the T4 primosome, which is composed of a DNA helicase (gene 41 protein) and a DNA primase (gene 61 protein)²⁶⁻³⁶.

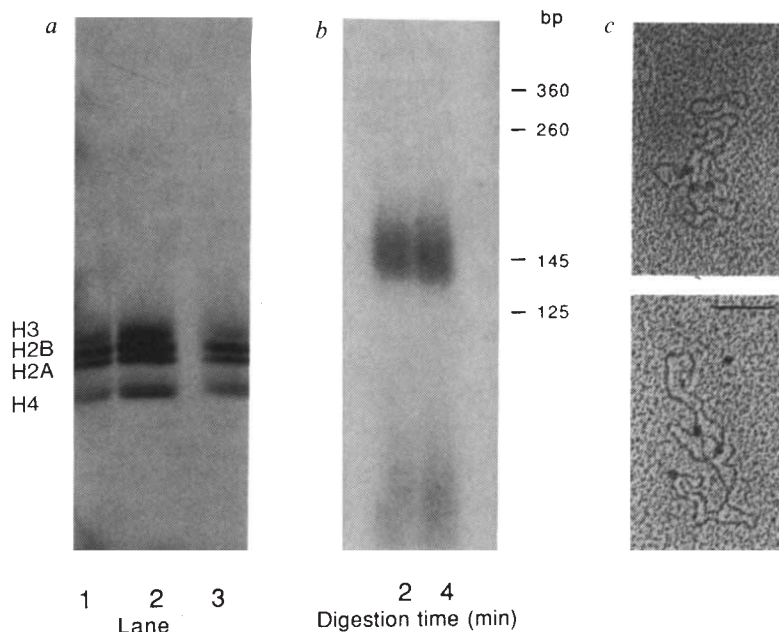
In our initial experiments, we found that the presence of nucleosomes on the DNA blocks replication catalysed by the T4 replication proteins. But nucleosomes can be bypassed by the T4 replication fork, provided that the T4 dda (DNA-dependent ATPase) protein is present. The dda protein is a DNA helicase that removes an RNA polymerase molecule or an operator-bound gene-repressor protein from the DNA template ahead of the fork^{23,37,38}. An additional T4 protein was included in the *in vitro* DNA replication system: the T4 59 protein. This protein, which has been recently purified and characterized, greatly facilitates the loading of an active primosome onto the replication fork, ensuring efficient lagging-strand, as well as leading-strand, DNA synthesis (J. Barry and B.A., manuscript in preparation).

On a circular template that lacks nucleosomes, replication proceeds in a 'rolling-circle' mode that allows many rounds of copying³⁹. Figure 2 shows that the leading-strand products are long DNA strands that move slowly during alkaline agarose gel electrophoresis, whereas the lagging-strand products accumulate as a heterogeneous smear of Okazaki fragments of smaller size (Fig. 2, third lane from left).

In the presence of nucleosomes, leading-strand DNA products of unusually short length were produced in the first few minutes

FIG. 1 Characterization of an artificial chromatin template. The plasmid pJMC110 DNA to which radioactive nucleosomes were transferred was purified by centrifugation through a sucrose gradient. After the peak fractions were pooled, concentrated, and dialysed into storage buffer, the nucleosomes on the DNA were characterized. *a*, Histone composition. Aliquots were subjected to SDS-PAGE on a 13.5% gel. Proteins were identified by autoradiography. Lane 1, initial H1-depleted native nucleosomes prepared from Chinese hamster ovary (CHO) cells; lane 2, nucleosome reconstituted on the DNA fragment containing the 5S rRNA gene (nucleosome donor); lane 3, nucleosomes on the artificial chromatin template (nucleosome acceptor). *b*, DNA protected from micrococcal nuclease digestion. After addition of CaCl_2 to 3 mM the artificial chromatin template was digested with micrococcal nuclease at 37 °C for 2 and 4 min. The DNA was then purified, end-labelled, and electrophoresed through an 8% polyacrylamide gel. The numbers to the right of the autoradiograph indicate the size of DNA fragment markers that were electrophoresed as standards in the same gel. *c*, Electron microscopy. The sample was fixed in 0.1% glutaraldehyde at 4 °C⁵⁷ and processed as described in ref. 58. Bar, 0.1 μm .

METHODS. To prepare a DNA template containing nucleosomes, labelled histone octamers were transferred to plasmid pJMC110 DNA by a low salt exchange method, as described in ref. 59. The histone donor was a nucleosome reconstituted on a 256-bp DNA fragment containing a sea urchin 5S rRNA gene which forms an exactly positioned core particle²⁴. Because of the unusual stability of the nucleosome on this DNA sequence, nucleosome dissociation, which leads to nonspecific binding of the histones to the acceptor DNA, was largely prevented during the exchange process. To prepare this histone donor, the 256-bp DNA fragment containing the 5S rRNA gene ($40 \mu\text{g ml}^{-1}$) was mixed with a 10-fold excess of H1-depleted nucleosome core particles⁵⁰, containing ^3H -labelled histone (prepared from ^3H -lysine-labelled CHO cells; core particle specific activity, $140,000$ c.p.m. μg^{-1}), at a DNA concentration of $400 \mu\text{g ml}^{-1}$ in a high-salt buffer (10 mM Tris-HCl buffer, pH 7.4, 1 mM Na_3EDTA , 2 mM DTT, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 0.8 M NaCl and $100 \mu\text{g ml}^{-1}$ human serum albumin (HSA)). Histone octamer exchange was performed by incubating this mixture for 20 min at 37 °C. The salt concentration was then decreased at 37 °C by two successive dilutions to 600 mM NaCl (100-mM steps every 10 min). The reconstituted nucleosome was then separated from the slower-sedimenting initial core particles by centrifugation through a sucrose gradient containing 0.55 M NaCl and $100 \mu\text{g ml}^{-1}$ HSA. The efficiency of reconstitution and purification was monitored by electrophoresis through a 6% polyacrylamide gel (acrylamide/methylene-bis-acrylamide ratio



of 46:1) as described in ref. 60. At least 90% of the DNA fragment containing the 5S rRNA sequence was reconstituted into nucleosomes, which contained the correct stoichiometry of the four core histones. Fractions containing these reconstituted nucleosomes were dialysed against 10 mM Tris-Cl buffer, pH 7.4, 1 mM Na_3EDTA , 2 mM DTT, 0.1 mM PMSF, 0.1 M NaCl and concentrated to $\sim 100 \mu\text{g ml}^{-1}$ DNA using a collodion bag apparatus. The reconstituted nucleosomes were stored at 4 °C in the presence of 0.02% sodium azide. No dissociation or proteolytic degradation of the histones was detected during these processes. The subsequent transfer of the histone octamers from the 5S rRNA gene to supercoiled plasmid pJMC110 DNA was performed by a similar method. The supercoiled DNA was incubated with the purified nucleosomes at molar ratios of 1:6 and 1:10 (plasmid:nucleosome). The exchange reaction was stopped by decreasing the salt concentration to 100 mM NaCl (100-mM steps every 10 min to 500 mM NaCl, followed by dilutions to 250 mM and 100 mM NaCl). The nucleosome-containing DNA molecule was then separated from the much smaller donor nucleosome either by centrifugation through a sucrose gradient or by chromatography on a Sepharose 4B gel filtration column in the presence of $100 \mu\text{g ml}^{-1}$ HSA.

of the reaction, indicating that the replication forks that had assembled moved more slowly than those that assembled when no nucleosomes were present on the DNA template. The replication forks were, however, able to pass a nucleosome after a pause. After 2 min of reaction, many of the replication forks had performed more than one round of synthesis on the chromatin template (leading strand >9.4 kb, see Fig. 2, rightmost lane), and by 5 min nearly all of the forks had done so (data not shown).

Histone octamers remain on the DNA

If the nucleosomes are knocked off the circular DNA template molecule every time the T4 replication fork passes, as is the *Escherichia coli* RNA polymerase molecule bound to a promoter²³, the leading-strand DNA polymerase molecule would encounter nucleosomes only during its first round of rolling-circle DNA synthesis (see Fig. 3a and below). In this case, all pausing of the replication fork on the nucleosome-containing template should disappear once the size of the leading strand reaches 9.4 kb (twice the size (4.7 kb) of the DNA circle). Examination of the time course of many experiments like the one of Fig. 2 reveals that strong pausing also occurs during the second round of rolling-circle DNA replication (leading-strand size between 9.4 and 14.1 kb). At least some of the nucleosomes must therefore remain on the DNA circle after the fork passes. But the extent of pausing clearly decreases as the leading strand grows, arguing against the model in which all of the histone octamers segregate as intact nucleosomes to the leading strand¹³.

A trivial interpretation of our results is that histone octamers are in fact knocked off the DNA by the moving fork, but very rapidly rebind to any DNA molecules in the solution (either as octamers or as smaller histone complexes). To test this hypothesis, we performed a series of *in vitro* replication experi-

ments in the presence of a large excess of nonreplicating DNA that lacked nucleosomes. If nucleosomes transiently dissociate, then this excess DNA should act as a 'trap' and rebind the dissociated histones, thereby altering their distribution. In this way, we could determine whether histone octamers are directly inherited by the daughter DNA molecules at the fork, or whether they transiently become free in solution. Under the physiological salt conditions (120 mM potassium acetate) that we used in these replication experiments, nucleosomes on the chromatin template do not transfer onto the added DNA spontaneously⁷.

We designed the experiment illustrated diagrammatically in Fig. 3a to test whether ³H-labelled histone octamers are released from an artificial chromatin template as a result of replication. Replicated DNA is readily discriminated from unreplicated DNA (including both unused chromatin templates and competitor DNA) by its resistance to the enzyme *Dpn*I. The replicated DNA molecules are separated from the digested unreplicated DNA on a Sepharose Cl-2B gel-filtration column. Figure 3b presents the result of such an experiment, performed both with and without a 10-fold excess of competitor DNA present during the DNA replication process. Experiments in which the replicated DNA was labelled with [³²P]dTTP show that all of the replicated molecules fractionate into the excluded peak (fractions 23–26). A large peak of ³H-labelled histones coeluted with this *Dpn*I-resistant newly synthesized DNA; the rest of the histones eluted later from the column, as expected from their presence on the substantial fraction of unreplicated DNA circles. Most importantly, the amount of ³H-labelled histone that eluted with the replicated DNA was insensitive to the presence of a 10-fold excess of competitor DNA (Fig. 3b), demonstrating that the histones are not released even transiently from the DNA during the passage of the replication fork. As expected, when the T4 DNA polymerase was omitted in the

FIG. 2 The effect of nucleosomes on replication fork movement *in vitro*. DNA synthesis was performed using either naked DNA (two lanes on the left (–nuc)) or artificial chromatin (two lanes on the right (+nuc)) as the template. The radioactive DNA product strands were sized by alkaline agarose gel electrophoresis at 1 and 2 min after the start of DNA synthesis. The total nucleotide incorporation into DNA was reduced four-fold by the presence of nucleosomes; to facilitate comparison of product sizes, twice as much reaction mix was analysed for the two lanes on the right. The indicated position of the primer strand after one round of rolling-circle replication (9.4 kb pairs) was determined from the migration rates of DNA markers (lane M). Because the replication forks proceeded for only a short distance on the nucleosome-containing templates (+nuc), most of these forks had not yet initiated Okazaki fragment synthesis (see Fig. 5 for results after further incubation).

METHODS. To provide a primer to start DNA synthesis on the plasmid pJMC110 DNA template, the DNA was specifically nicked at the M13 bacteriophage gene 2 protein recognition site. One unit of bacteriophage fd gene 2 protein⁶¹ was incubated with 0.5 µg DNA in 20 mM Tris-HCl buffer, pH 8.5, 80 mM NaCl, 2.5 mM MgCl₂, 1 mM β-mercaptoethanol, 5% glycerol at 30 °C for 2.5 min. The reaction was terminated by addition of Na₃EDTA to 3 mM and cooling the reaction mixture to 4 °C. Analysis of the products of this reaction by agarose gel electrophoresis showed that ~90% of the supercoiled DNA was converted to open circular form in the reaction. But when a chromatin template was treated in this way, the percentage of open circular form produced was reduced if more than a few nucleosomes were reconstituted on each plasmid DNA molecule. *In vitro* DNA replication was performed in the presence of 33 mM Tris-acetate (pH 7.8), 120 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM DTT, 1 mM ATP, 1 mM GTP, 0.1 mM UTP, 0.1 mM CTP, 0.5 mM dATP, 0.5 mM dGTP, 0.15 mM dCTP, 0.08 mM [α-³²P]TTP, 5 µg ml⁻¹ template DNA, in the presence of 100 µg ml⁻¹ nuclease-free HSA as a protein carrier. In these reactions, the following T4 DNA replication proteins were present at the indicated concentrations: 2 µg ml⁻¹ T4 DNA polymerase, 50 µg ml⁻¹ T4 gene 32 protein, 20 µg ml⁻¹ T4 gene 44/62 protein, 18 µg ml⁻¹ T4 gene 45 protein, 20 µg ml⁻¹ T4 gene 41 protein, 1 µg ml⁻¹ T4 gene 61 protein, 10 µg ml⁻¹ T4 dda protein and 1 µg ml⁻¹ T4 59 protein. Partial synchronization of the replication reactions on the specifically nicked plasmid pJMC110 DNA tem-

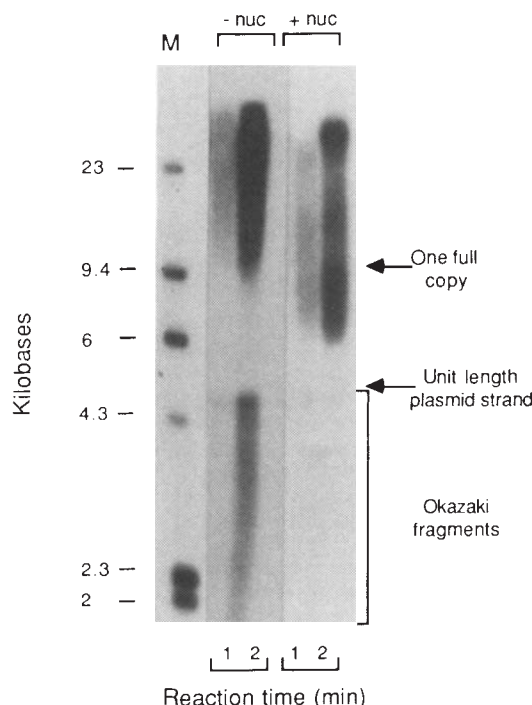


plate was achieved by preincubation in the absence of dCTP before allowing extensive DNA synthesis to begin by adding dCTP. The radioactive products of the reaction were then analysed by separating them by electrophoresis through a 0.6% agarose gel run in 30 mM NaOH, 1 mM Na₃EDTA, and autoradiographing the dried gel²³.

replication reaction (dotted line), ~95% of the ^3H -labelled histones coeluted with the small DNA fragments produced by *Dpn1* treatment (Fig. 3b, fractions 32–60).

Table 1 presents the results from several experiments identical to the experiment of Fig. 3b. In general, 30–35% of the ^3H -labelled histone radioactivity was found associated with newly synthesized DNA in fractions 23–26, and the competitor DNA had no effect on this result. We conclude that among the DNA template molecules nicked by treatment with the gene 2 protein (~50% of the DNA templates), most, but not all, are used as a template by the T4 replication proteins.

We next examined whether all four of the individual histones remain associated with the replicated DNA molecules. For histones H_2B , H_2A and H_4 , the ratio of histones was the same as before replication (Fig. 4a; unfortunately, although H3 was present, its specific activity was too low to detect it accurately by fluorography). In addition, we digested the purified replicated DNA with micrococcal nuclease to test for DNA that was resistant to digestion because of the presence of nucleosomes. Protected DNA fragments with a size identical to that of fragments protected in a nucleosome core particle were observed (Fig. 4b). With further digestion, DNA fragments of a smaller size appeared, which were due to internal cuts in the nucleosome by micrococcal nuclease (lane 5).

We also attempted to examine the effect of replication on the structure of the nucleosome by electron microscopy. The electron micrographs indicated that histones that are bound to replicated DNA retain the basic nucleosomal structure. Globular particles morphologically similar to native nucleosomes were found associated with both the long double-stranded DNA tail

TABLE 1 Effect of 10-fold excess competitor DNA on retention of histones

Experiment	% Newly made DNA in peak	% Histone in peak 1 (no DNA competitor)	% Histone in peak 1 (+DNA competitor)
1	98	—	—
2	—	35	30
3	—	30	32
4	—	30	35
5	—	—	30

A 10-fold excess of competitor DNA has no effect on the retention of histones on replicated DNA molecules. DNA synthesis was allowed to occur for 5 to 10 min. The procedure described in Fig. 3a was then used to separate replicated DNA molecules, as in Fig. 3b. The *Dpn1*-resistant DNA fractions in peak 1 contain replicated DNA molecules, which produced an average of 3–4 template copies by rolling-circle replication (see Fig. 3a). Either ^{32}P -labelled DNA or ^3H -labelled histones were monitored, depending on the component that was radioactive. The presence of the 10-fold excess of competitor DNA did not affect the efficiency or rate of replication of the chromatin template (data not shown).

and with the circular template (Fig. 5a). But we did not find this method to be a good way of analysing nucleosome behaviour during replication, because only a very small fraction of the replicated molecules were sufficiently spread for scoring. In addition, a significant amount of histone octamer dissociation occurred during the preparation steps required for electron microscopy.

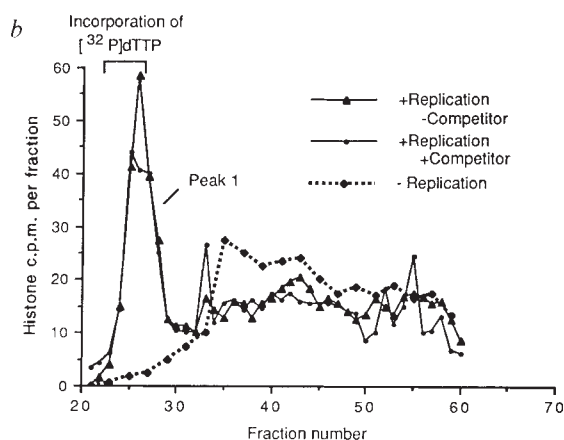
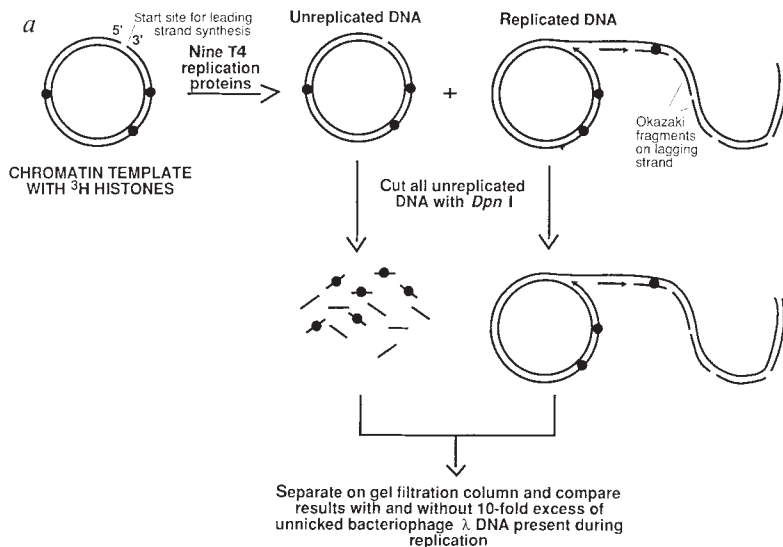


FIG. 3 The use of competitor DNA ($83 \mu\text{g ml}^{-1}$) to test for the direct transfer of histones from parental to daughter DNA helices at a DNA replication fork. DNA synthesis was performed with the complete T4 DNA replication system containing the nine proteins, as described in the text (see also Fig. 2). a, Schematic diagram of the experiments. In rolling-circle DNA replication, the leading strand DNA polymerase molecule moves continuously around the unnicked strand of the original circular DNA template molecule. As it moves, a single strand of DNA is displaced from the circle as a long linear 'tail'. All of the DNA product on the tail is synthesized by the lagging-strand DNA polymerase molecule, which produces a series of Okazaki fragments that remained unsealed in our experiments. In the T4 system, these fragments are very heterogeneous in length (400–4,000 nucleotides)²⁵. Because the plasmid pJMC110 DNA was produced in *dam*⁺ bacteria, it contains 22 fully methylated GATC sequences, which are cut by *Dpn1*. The hemimethylated DNA produced by replication *in vitro* becomes resistant to *Dpn1* cleavage⁶². After *Dpn1* treatment, the replicated molecules are separated from the *Dpn1*-degraded unreplicated plasmid DNA by chromatography on a Sepharose Cl-2B column. To test for the dissociation of labelled histones from the nucleosome-containing DNA template during replication, a 10-fold excess of linear DNA molecules was added as competitor to rebind any histones released during the replication reaction. This competitor DNA was

prepared by digesting bacteriophage λ DNA with *Bst*Ell and *Eco*RV, which produces fragments of 1.0 to 3.5 kb that do not replicate and can be readily separated from the much larger replicated molecules by Sepharose Cl-2B gel filtration. b, Gel filtration analysis of DNA-bound ^3H labelled histones after replication of the artificial chromatin template in the presence or absence of competitor DNA. Replication was performed with $1 \mu\text{g}$ template and $10 \mu\text{g}$ competitor DNA in a total volume of $120 \mu\text{l}$. After 10 min of replication at 37°C , the salt concentration was raised to 190 mM NaCl . Twenty units of *Dpn1* were then added for 20 min at 37°C . After addition of $20 \text{ mM Na}_3\text{EDTA}$, the reaction products were chromatographed on a Sepharose Cl-2B gel filtration column ($30 \times 0.5 \text{ cm}$) run at a flow rate of 0.5 ml h^{-1} in Tris-HCl buffer, pH 7.5, $1 \text{ mM Na}_3\text{EDTA}$, 10 mM NaCl at room temperature. Elution is from left to right, with fraction volumes of 0.15 ml . Aliquots from each fraction were counted to determine the distribution of the ^3H radioactivity. In control experiments, an aliquot of the same chromatin template replicated in the presence of ^{32}P -labelled TTP was analysed to determine the elution profile of newly replicated DNA (marked with a bracket). The dotted line shows that no histones appear in peak 1 if DNA replication is blocked by omission of the T4 DNA polymerase (gene 43 protein) from the reaction mixture.

TABLE 2 Quantitation of electron-microscopy data

Size of single-stranded bubble (nucleotides): from ref. 22, 160 ± 35 ; unreplicated, 147 ± 37 ; replicated circle, 150 ± 35 ; replicated tail, 145 ± 30 .

Partitioning of nucleosomes between circle and tail

No. of template copies	No. of DNA molecules	Total nucleosome no. on circle	Total nucleosome no. on tail	Total nucleosome ratio tail: circle*	Average no. of nucleosomes per circle	Average no. of nucleosomes per tail
0.6-1	6	26	8	0.30 (0.25)	4.3	1.3
1-2	15	28	17	0.6 (0.52)	1.8	1.1
2-3	13	20	19	0.95 (1.0)	1.5	1.5
3-4	5	5	14	2.80 (1.7)	1.0	2.8
>4	7	5	31	6.20 (>2.2)	0.7	4.4

The quantitation is of data presented in Fig. 6.

* The numbers in parentheses denote the nucleosome ratio expected if the nucleosome has a 0.75 chance of segregating to the leading strand and a 0.25 chance of segregating to the lagging strand during each pass of a replication fork.

Nucleosome segregation at the replication fork

To permit a quantitative analysis of nucleosome behaviour in the *in vitro* DNA replication system that we used, we directly photo-crosslinked the replication products containing nucleosomes with psoralen, and examined them by electron microscopy under conditions that normally separate the DNA strands. This method has been used previously to probe chromatin structure^{22,40-42}. As expected, when we spread the chromatin templates under these denaturing conditions, they displayed single-stranded bubbles of the size reported for DNA organized with histones in nucleosome structure (Fig. 5b); no such bubbles were observed for the same DNA molecules lacking nucleosomes (data not shown). The chromatin templates used for this experiment were in this way determined to contain an average of three to four nucleosomes.

After replication, the application of this technique revealed rolling-circle DNA molecules with long tails (see Fig. 3a). Both the circle and the tails contained occasional bubbles where a nucleosome had protected the DNA from crosslinking by psoralen (Fig. 5c). These bubbles were of the size expected for nucleosomes (Table 2); as expected, they were only detected when nucleosome-containing DNA molecules were used as the template (data not shown).

We mapped the locations of the bubbles for 46 replicating DNA molecules; the nucleosome distributions inferred are shown in Fig. 6. This figure shows that those molecules that replicated the most extensively tend to have the fewest nucleosomes on their DNA circle and the most on their tail. Because the tail represents the DNA made on the lagging strand of a replication fork, this is the result expected if a nucleosome partitions more or less randomly between the two daughter DNA helices produced at the fork each time that the fork passes.

The 'partition coefficient' for a nucleosome is examined more quantitatively in Table 2 in which the molecules represented in Fig. 6 have been divided into five classes on the basis of the extent of their replication. Because the average number of nucleosomes per DNA molecule does not decrease with an increase in the extent of replication, and is consistent with the number determined for the unreplicated template, we can conclude that most of the histone octamers were inherited intact on the daughter DNA helices. We tabulated the ratio of nucleosomes on the tail to nucleosomes on the circle for each class of replicated DNA molecules (Table 2). Comparison with the theoretical result expected for various partition coefficients indicates that a nucleosome most often segregates to the leading strand, but that it segregates to the lagging strand about one in every four passes of a replication fork (see numbers in parentheses in Table 2). It seems that the choice is made randomly, as it is clear from the presence of nucleosomes all along the DNA tails that the same nucleosome can partition to the leading strand during one pass of the replication fork and then partition to the lagging strand at the next pass of the same replication fork a few minutes later.

Discussion

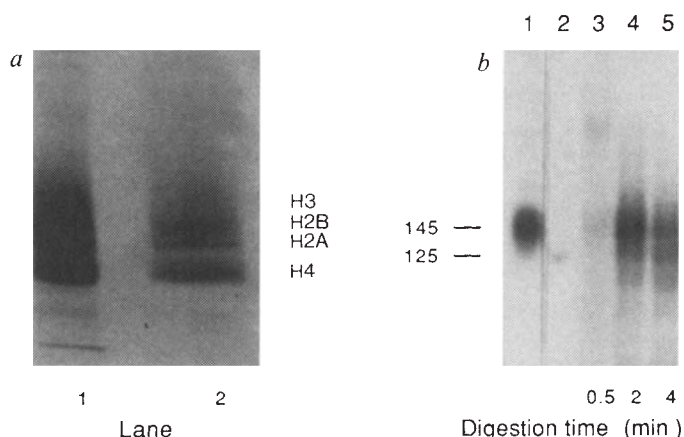
The choice of replication system and DNA template. We have described here the use of a well-characterized purified prokaryotic DNA replication system to study the behaviour of an important eukaryotic structure, the nucleosome, at a replication fork. The replication proteins used form a multienzyme complex that catalyses concerted leading- and lagging-strand DNA synthesis with *in vivo* rates and fidelities²⁵. The choice of a hybrid prokaryotic-eukaryotic system was necessary for our experiments, because no highly purified eukaryotic DNA replication system has so far been developed. But because the system is completely defined, it has the valuable feature of demonstrating that the observed nucleosomal properties are inherent to the nucleosome itself, rather than being due to hypothetical replication proteins that stabilize the histone octamer and tether it to the DNA molecule, thereby preventing its release as the DNA polymerase passes.

By using a specifically nicked circular DNA molecule to produce the artificial chromatin template, we generated replication forks on a high proportion of the template molecules; moreover, once started, these replication forks proceeded around the circle indefinitely, generating the long linear tails diagnostic of the rolling-circle form of DNA replication³⁹. A unique advantage of this form of replication is that the extent of replication on each template molecule is readily determined from the tail length measured by electron microscopy. In addition, because the same replication fork passes repeatedly over the daughter DNA helix synthesized on the leading side of the fork, the tail is formed entirely by lagging-strand DNA synthesis. It is this feature of DNA synthesis on a circular template that allowed us to determine how the nucleosome is partitioned between the two daughter DNA helices produced by the replication fork (Fig. 6; Table 2).

The histone octamer is directly inherited by the daughter DNA helices produced at a replication fork. The ³H-labelled histones in template-bound nucleosomes co-fractionated with extensively replicated DNA molecules in a manner that is unaffected by the presence of a 10-fold excess of nonreplicating competitor DNA (Fig. 3b and Table 1). Moreover, these histones seem to form normal nucleosomes on the daughter DNA helices: the inherited unit contains all four histones (Fig. 4a), protects ~145 bp of DNA against micrococcal nuclease digestion (Fig. 4b), and masks the same length of DNA against psoralen cross-linking as is masked by native nucleosomes (Table 2). We conclude that nucleosomes can undergo conformational changes that allow replication directly through them. Nucleosomes in solution can adopt different structural states^{43,44}, and drastic reversible conformational changes of histone-DNA complexes have been observed by electron microscopy^{42,45}.

Nucleosomes allow *in vitro* transcription to pass them^{46,47}. But Lorch *et al.*⁴⁶ reported that RNA polymerase displaces the histone octamer from the DNA, whereas Losa and Brown⁴⁷ suggest that the histone octamer remains at its initial DNA site

FIG. 4 Characterization of the histones that segregate with replicated DNA in the presence of a large excess of competitor DNA. *a*, Fluorograph of the ^3H -labelled histones that elute in peak 1 from a Sepharose 2B gel filtration column. To obtain enough radioactivity for this experiment, the peaks from five separate experiments like that represented in Fig. 3*b* were pooled (lane 2). Because the histones were labelled by the incorporation of ^3H -labelled lysine, they had a low specific activity and were relatively difficult to detect. For comparison, lane 1 displays the histones present on the artificial chromatin template before replication. *b*, Analysis of the DNA protected from micrococcal nuclease digestion in the purified replicated chromatin. The material isolated in peak 1 was digested with micrococcal nuclease for the indicated times and its size then analysed by polyacrylamide gel electrophoresis (lanes 3–5). For comparison, the DNA protected from digestion in native core nucleosomes was similarly analysed in lane 1. Lane 2 displays the migration rate of a 125-bp DNA fragment that served as a size marker.



after the RNA polymerase passes. It has previously been suggested that dissociation into half nucleosomes⁴⁸ or 'lexosomes'⁴⁹ accompanies transcription.

During DNA replication, the entire histone octamer is segregated to only one of the two DNA daughter helices. This implies that the nucleosome core becomes transiently bound to a DNA single-strand, as previously suggested^{7,50}. Histone-DNA interactions that are different from those in normal nucleosomes could exist that allow the histones to remain bound transiently to a single DNA strand without covering the nucleotide bases

or interfering with the replication process. Alternatively, the histone octamer could somehow 'rock' back and forth as the DNA polymerase passes, allowing the DNA polymerase to copy a histone-free region of DNA without knocking off the nucleosome completely. In either case, once the replication fork has passed, the histone octamer must re-form a normal nucleosome.

The positions of all of the nucleosomes left on the circular portion of the rolling-circle DNA products in the experiment of Fig. 6 were determined by electron microscopy (data not shown). There was no tendency for these nucleosomes to

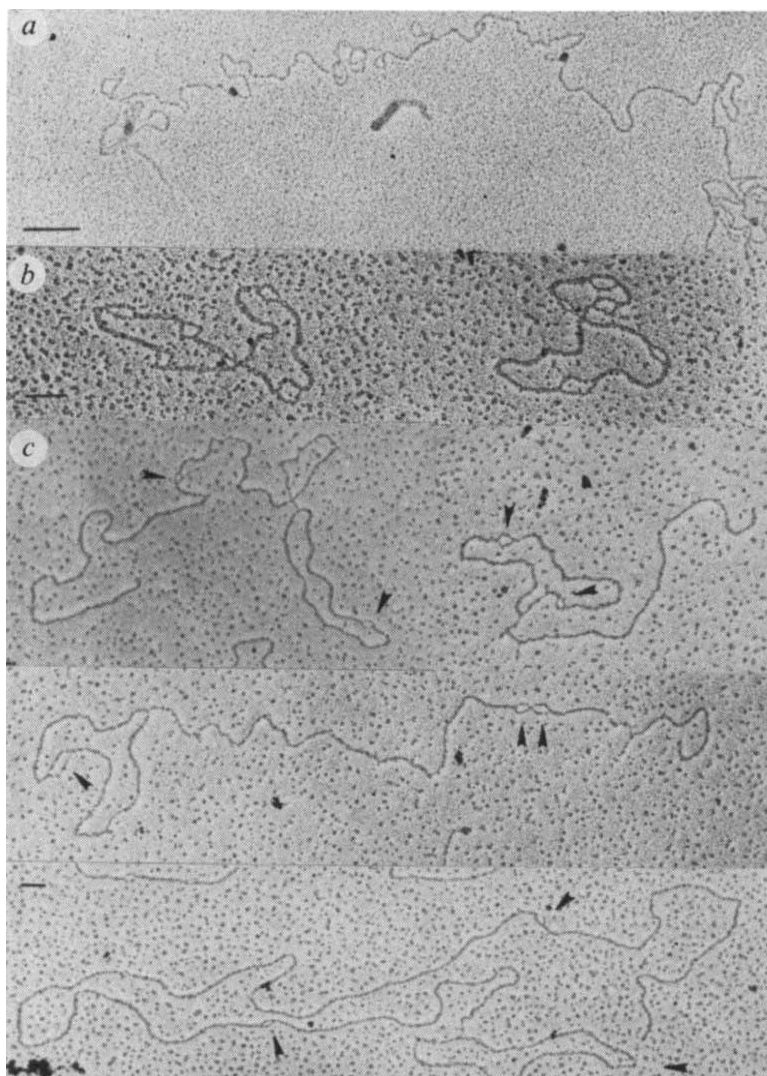


FIG. 5 Electron micrographs of the artificial chromatin template before and after replication. *a*, Replicating molecules spread as chromatin, where nucleosomes appear as small beads (scale bar, 0.1 μm). *b*, Unreplicated chromatin template molecules viewed by the psoralen technique. The positions of nucleosomes appear as single-stranded bubbles in the deproteinized DNA (bar, 0.1 μm). *c*, Four replicating molecules viewed by the psoralen technique. Bubbles representing nucleosomes (arrows) are found on both the DNA circle and its tail (bar, 0.1 μm).

METHODS. The procedure for psoralen cross-linking of H1-depleted chromatin was essentially as described in ref. 63. Samples were mixed with 0.03 $\mu\text{g ml}^{-1}$ psoralen and then irradiated with ultraviolet light (360 nm) at 4 $^{\circ}\text{C}$ for 65 min (Model UVGL-25 UVP-incorporated lamp) at a distance of ~ 2.5 cm. The DNA was then deproteinized by treatment with proteinase K (200 $\mu\text{g ml}^{-1}$) in the presence of 0.5% SDS for 2 h at 37 $^{\circ}\text{C}$, followed by two phenol-chloroform extractions and ethanol precipitation. The psoralen-crosslinked DNA was resuspended in 10 mM Tris-HCl buffer, pH 7.4, 1 mM Na_2EDTA , and incubated for 60 min in a denaturing buffer containing 73% (v/v) formamide, 0.5 M glyoxal (Kodak), which separates all noncrosslinked DNA strands. This DNA was then viewed by electron microscopy after spreading, as described in ref. 64.

accumulate near where the tail joins the circle (the fork location). Such an accumulation would be expected if the template-bound nucleosomes tend to be pushed along the DNA template as the replication fork approaches (nucleosome sliding). But whether or not each nucleosome remains on exactly the same DNA sequence after the fork passes cannot be determined from our present results. Further studies using our *in vitro* system should, however, enable us to address this question.

The parental histone octamer can be transferred to either of the daughter DNA helices when the fork passes. One side of a replication fork (the lagging strand) contains a single-stranded region of the template not present on the other side (the leading strand). At the T4 replication fork, this single-stranded connection to the parental double helix is often $>1,000$ nucleotides, and it is normally covered with cooperatively bound gene 32 protein molecules²⁵. Intact histone octamers can nevertheless be transferred to the DNA helix made on the lagging strand (the tail in Fig. 6). In one variation of the experiment of Fig. 3a, the primase (gene 61 protein) was omitted from the replication reaction so that DNA was synthesized only on the leading side of the fork. In this case, a long length of single-stranded DNA accumulated on the rolling-circle tail because no Okazaki fragments were made. When peak 1 from a gel filtration column like that represented in Fig. 3b was analysed, very little ³H-labelled histone was found attached to this replicated DNA (data not shown). This result indicates that the histone octamers that would normally end up on the lagging-strand DNA helix were transferred to the long single-stranded tail instead, where they are relatively unstable⁹, and so dissociate during the procedures used to isolate peak 1. Therefore, transfer of a parental histone octamer to the lagging strand does not seem to require the DNA to be double-stranded. We suggest that the normal transfer of the histone octamer to the lagging strand involves a similar unstable intermediate, but because each cycle of Okazaki fragment synthesis requires only a few seconds^{25,51}, the histone octamer survives to become stabilized as a normal nucleosome on double-stranded DNA.

The presence of an unstable intermediate during nucleosome transfer from parental to daughter DNA can explain why the regions of newly synthesized DNA *in vivo* (as measured by pulse-label incorporation of ³H-labelled thymidine) have sometimes been found to be free of nucleosomes after chromatin extraction^{15,52,53}. In most of these experiments, the chromatin was washed in 200 mM NaCl before analysis, and there is evidence that the histones on newly replicated DNA are unusually sensitive to salt-induced dissociation⁵⁴. In addition, Sogo *et al.*²² failed to detect nucleosomes both just in front of the replication fork and just behind it when the psoralen cross-linking technique was applied to SV40 chromatin replication

in vivo. Although their results were interpreted as indicating that the histone octamer is released from the DNA as the replication fork approaches, our results support the alternative view that the histones remain on the DNA, but are present in an altered more-open complex that leaves the DNA accessible to psoralen crosslinking⁴². The nature of this altered form of the nucleosome could be susceptible to analysis in our *in vitro* system.

The partition coefficient for histone octamers between leading- and lagging-strands. Our results indicate that although the histone octamers ahead of the fork can remain associated with either of the strands at the replication fork, each shows a bias of about 3 to 1 towards remaining on the leading strand (Fig. 6 and Table 2). Given the inherent difficulty of analysing the process *in vivo*, it is perhaps not surprising that the results of direct nucleosome segregation studies on cells have been the subject of intense controversy. Most recent studies tend to support the claim that the parental histones are distributed to both sides of the replication fork *in vivo*². But our *in vitro* results are in agreement with those of a recent study¹⁴ indicating that the segregation of nucleosomes is biased in favour of the leading strand in cycloheximide-treated mammalian cells (in these studies, as in ours, no new nucleosomes were deposited behind the fork).

Our *in vitro* system is not a perfect mimic of the eukaryotic replication fork moving through native chromatin. First, the artificial chromatin template that we used contains only about one nucleosome per kb, a five-fold lower density of nucleosomes than is present *in vivo*. Second, the nucleosomes that we used were randomly distributed along the DNA template, rather than organized into regular repeated arrays with the aid of histone H1. Third, it is possible that special eukaryotic proteins—either replication proteins or non-histone proteins with a structural role—influence the behaviour of the histone octamer at the fork, perhaps even causing different patterns of nucleosome segregation in different regions of the genome. Finally, in eukaryotic cells the Okazaki fragments (and therefore the single-stranded regions of template on the lagging strand) are much shorter than they are in our prokaryotic replication system (averaging 200 nucleotides rather than $\sim 1,500$ nucleotides), and the T4 gene 32 protein is, of course, not present on the single-stranded regions of the lagging-strand. In our view, these differences could affect the partition coefficient for a nucleosome between leading and lagging strands, but they are unlikely to change the basic conclusions derived from our *in vitro* study.

Conclusions

Our main conclusions are that the histone octamers remain bound on the DNA template when a replication fork passes and that they are designed in a way that allows them to transfer

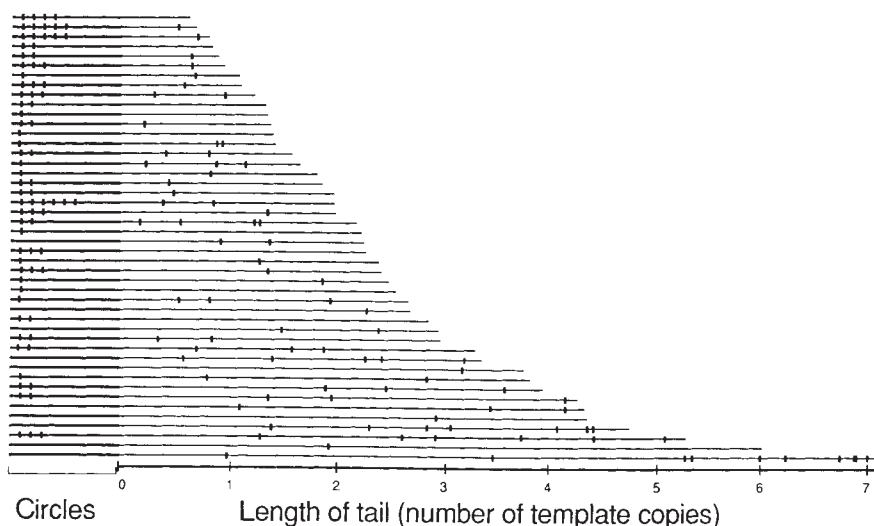


FIG. 6 Histogram showing the positions of nucleosomes on 46 replicating DNA molecules, measured from micrographs like those shown in Fig. 5c. The artificial chromatin template circles used for this experiment contained an average of three to four nucleosomes. Replication was for 10 min at 37 °C. The short vertical bars show where the centres of nucleosomes were located on the tails of each rolling-circle molecule; by contrast, the bars on the circular portions of each molecule denote the number, but not the position, of nucleosomes. For a schematic diagram of these replicating molecules, see Fig. 3a.

efficiently to either one of the two daughter DNA helices produced by the fork. Because the basic mechanisms of DNA replication and the geometry of the replication fork seem to be identical for prokaryotes and eukaryotes⁵⁵, the results that we obtained with the T4 bacteriophage model system have important implications for eukaryotic replication. Even though replication over nucleosomes would seem to demand significant conformational changes in the histone octamer, the observation that it can be achieved without eukaryotic proteins demonstrates

that histones are especially designed to facilitate this important biological process. We suggest that the striking evolutionary conservation of the histone amino-acid sequences, as well as the construction of the nucleosome core from eight small histone molecules, reflects the need for transient nucleosome structures that allow the DNA in eukaryotic chromosomes to be condensed and packaged without interfering with either DNA replication or transcription. □

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We dedicate this paper to the memory of Abraham Worcel; his death on 27 December 1989 leaves the field of chromatin research without one of its major contributors.

LETTERS TO NATURE

Large-scale distribution of galaxies at the Galactic poles

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GALAXIES, mapped in two or three dimensions, are not distributed randomly but are clustered on small scales ($<5 h^{-1}$ Mpc, where $h \approx 0.5$ –1 is Hubble's constant in units of $100 \text{ km s}^{-1} \text{ Mpc}^{-1}$), for reasons conventionally ascribed to the effects of gravity. Whether galaxies remain correlated on very large scales (~ 50 – $100 h^{-1}$ Mpc) is of particular interest, because such structures are unexpected in most cosmological theories. We have combined data from four distinct surveys at the north and south Galactic poles to produce a well sampled distribution of galaxies by redshift on a linear scale extending to $2,000 h^{-1}$ Mpc. Here we report our finding

of an excess correlation and an apparent regularity in the galaxy distribution with a characteristic scale of $128 h^{-1}$ Mpc. This structure is revealed only after the completion of recent surveys extending to redshift $z > 0.2$. Similarly deep surveys with greater angular spread are needed to verify our results and to determine the implications for cosmology.

Recently there has been a considerable increase in information on the three-dimensional distribution of galaxies from large systematic redshift surveys. These surveys have generally been of two kinds. First, there are wide-angle shallow surveys¹ in which information on the clustering is affected by problems of non-uniform detection and questions of whether the local volumes probed are representative. Second, there are deeper narrow-cone surveys in a few directions^{2–4}; these overcome such problems to some extent but require more telescope time. Recently, through the advent of multiple-object spectroscopy, some very deep pencil-beam surveys have been attempted^{5,6}, the aim of which is to study collectively galaxy evolution and clustering on substantially larger scales than hitherto possible.

Much of the pencil-beam work has concentrated at the two Galactic poles, where effects of obscuration should be minimal. A deep-redshift survey recently completed at the Anglo-Australian telescope (AAT) sampled five southern high-latitude fields of 20-arcmin diameter to a limiting photographic magnitude $b_1 = 21.5$ (ref. 5) ($b_1 = \text{Kodak IIIa-J} + \text{Schott GG385}$). Independently, at Kitt Peak, a second long-term redshift survey

is continuing which samples 5-arcmin zones in various high-latitude northern fields each typically 40 arcmin across in the magnitude range $b_1 < 22.5$. The surprisingly clustered nature of the galaxy distributions found in such surveys has already been noted, but not analysed⁶.

The typical deep survey field is $5 h^{-1}$ Mpc across at the mean depth of $z \sim 0.2$. This is small compared with the survey depth, but is greater than the core radius of a typical rich cluster ($\sim 0.1 h^{-1}$ Mpc). In all four fields for which there is adequate data for examining structures on large scales (> 70 redshifts)—three from the Kitt Peak survey, one from the AAT survey—reasonably strong positive correlations are found at separations of $\sim 100 h^{-1}$ Mpc. This signal arises from the surprisingly regular spacing of peaks in the redshift distributions. Because the southern and northern Galactic polar fields (SGP, NGP) are almost exactly opposite (to within 0.4°), here we combine the data to examine the distribution on a single axis of extent $\sim 2,000 h^{-1}$ Mpc (Fig. 1a). Remarkably, a single regular distribution with constant comoving pair separation of $128 h^{-1}$ Mpc (adopting a deceleration parameter $q_0 = 0.5$) provides an accurate representation of most of the strong features.

Selecting the polar fields has the further advantage that these fields have been probed with a larger solid angle in a shallower-redshift survey^{2-4,6}. For the SGP, the bright survey has the same field centre to within 0.3° and provides valuable data with $z < 0.1$ where the deep survey is sample limited (Fig. 1b). There is some evidence for a similar regularity. A further bright southern survey, consisting of two fields SP3 and SP4 in refs 3, 4, also shows a redshift distribution structure of a similar kind but the peaks move slightly in redshift. These field centres are 5° and 4° away, respectively, from the pole.

For the NGP, a bright northern survey (field NP5 in refs 3, 4) is also equivalent to its southern counterpart sampling $z < 0.15$; its field centre is displaced by 7° from the deep equivalent. This survey displays two prominent overdensities separated by a large interval intersecting the periphery of the Böotes void⁷. As in the south, the precise redshift of these overdensities is dependent on sky position; a further bright field (NP4) with a centre 13° away from the deep field shows the first peak shifted marginally to a lower redshift ($z = 0.023$, Fig. 1b). This closest northern peak can be identified with galaxies associated with the Coma supercluster⁸.

Three of the four surveys in Fig. 1 are strictly magnitude limited and statistically complete. The northern deep survey has a sampling rate that depends on apparent magnitude but its selection function is reasonably close to a magnitude-limited sample. The redshift precision is in each case better than the bins used in Fig. 1. Redshifts for the survey galaxies were generally determined independently using both emission and absorption line features, so there is little possibility of any bias leading to false repetition in the extraction of redshifts from the spectra. Further, entirely different groups and instruments have been used yet the regularity is seen in both hemispheres, and the effect is strengthened when the datasets are combined. The combined data in Fig. 1 indicates a remarkably regular one-dimensional distribution with most galaxies lying in discrete peaks separated by $128 h^{-1}$ Mpc.

It is important to preface any statistical significance estimates with the admission that these are *a posteriori* calculations. A one-dimensional autocorrelation and power-spectrum analysis of all the redshift data available along the NGP-SGP axis (Fig. 2) show that the smallest peak separation of ~ 128 Mpc seems to be a fundamental mode of a remarkably regular distribution extending out to pair separations of at least $1,000 h^{-1}$ Mpc. To assess the significance of our results, we assume as our null hypothesis a model with no large-scale power beyond the already well-known small-scale galaxy correlations described by the two-point correlation function, $\xi(r) = (r/r_0)^{-1.8}$. This small-scale clustering will cause an excess variance in the cell counts, which also affects the fluctuations in the large-scale correlation

function. We calculate this effect by approximating the combined selection functions with a narrow cylinder of radius R and length D . For a cylindrical bin of radius R , length L in a survey of N galaxies, the mean small-scale correlation is integrated numerically: $\xi_0(R, L) = 2.24 r_0^{1.8} L^{-0.75} R^{-1.05}$ and the variance of the pair counts in one bin is

$$\langle (\delta n_p / n_p)^2 \rangle = 4 \left(\frac{1}{N} + \xi_0 \frac{L}{D} \right).$$

For our combined survey, we use $r_0 = 5 h^{-1}$ Mpc (ref. 9), $R = 3 h^{-1}$ Mpc, $D = 2,000 h^{-1}$ Mpc, $L = 30 h^{-1}$ Mpc and $N = 396$; thus the standard deviation of the pair count in a given bin becomes ≈ 0.26 . The excess correlation in the first and second peaks of Fig. 2a are thus each a $4-6\sigma$ effect. A similar significance can be determined from the height of the peak in the power spectrum in Fig. 2b. Under the null hypothesis of no large-scale power, the amplitude distribution is exponential and, with reference to the r.m.s. noise in the spectrum, the peak has a formal probability of $\sim 3 \times 10^{-4}$.

It should be realized that the power spectrum in one dimension is a projection of that in three dimensions and on large scales we cannot therefore attach great significance to the particular scale $128 h^{-1}$ Mpc (which will in general be greater than that for a corresponding three-dimensional feature) without a better understanding of the spatial form of the large-scale structure. For this reason it is not necessarily appropriate to co-add signals from sparsely sampled fields in other directions. The present data imply the existence of structure on scales much larger than

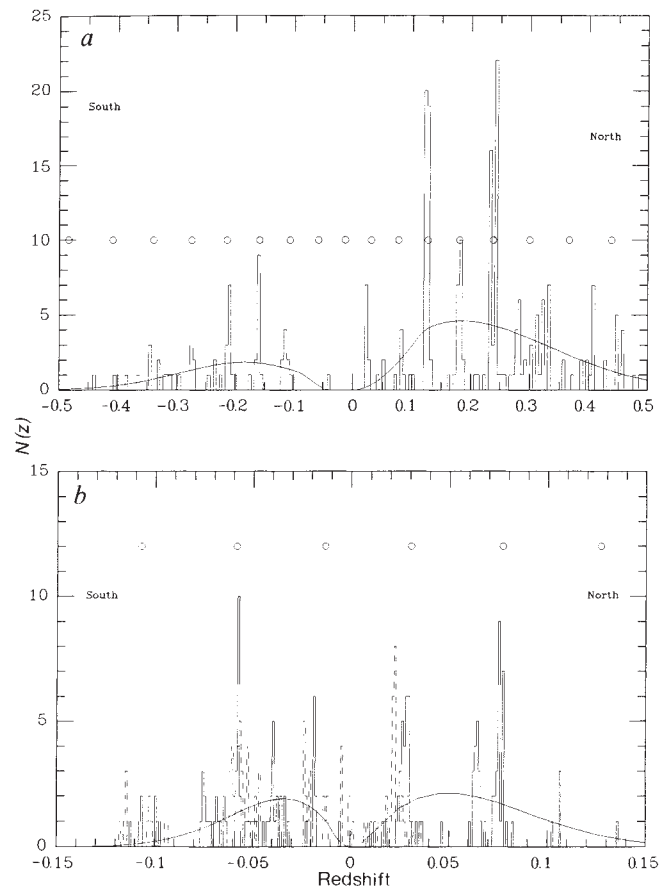


FIG. 1 Redshift distribution for four surveys at the Galactic poles with selection functions indicated as solid curves and normalized to each data set. Solid histograms refer to fields close to each other in position; dashed histograms refer to more widely distributed fields (SP3 and SP4 in the south, NP4 in the north^{3,4}). *a* refers to faint samples^{5,6} and *b* to bright surveys²⁻⁴. Circles indicate a best-fit constant comoving separation of $128 h^{-1}$ Mpc for $q_0 = 0.5$.

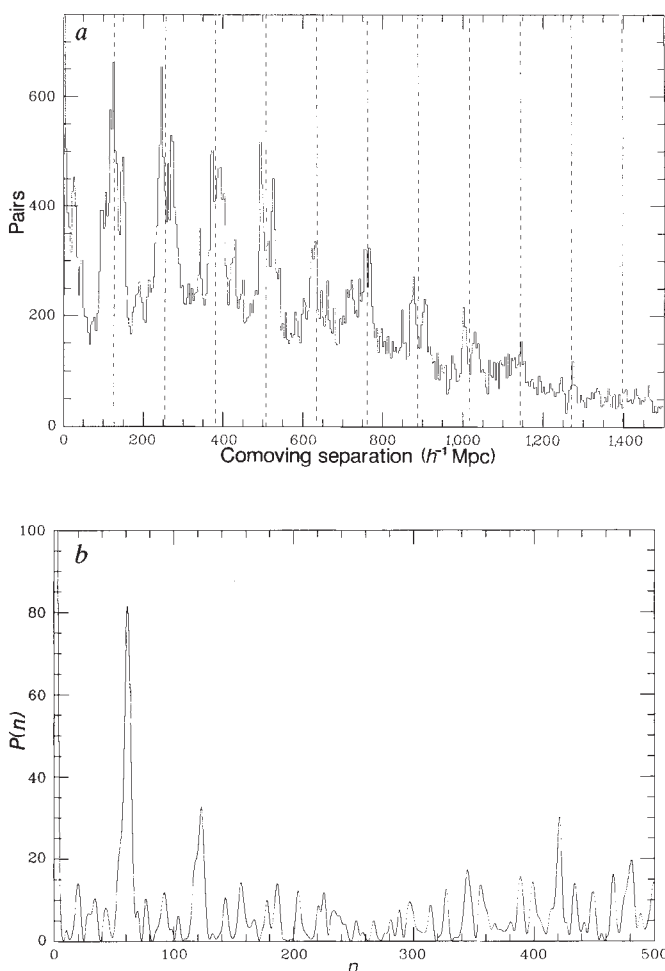


FIG. 2 *a*, One-dimensional pair-count correlation for all the redshift data available in the SGP-NGP axis. The dashed line indicates scales as multiples of $128 h^{-1} \text{ Mpc}$. *b*, Power spectrum analysis of the same dataset, where $r(n) = 8,000/n h^{-1} \text{ Mpc}$.

previous estimates. Indeed this scale is too large to have been adequately sampled by previous redshift surveys. Preliminary deep-survey data in other directions (SA68 and Hercules) also indicate power on a similar scale.

Our results are tentative at present, and possibly unappealing in terms of standard cosmologies. Highly structured redshift distributions are claimed to be consistent with one-dimensional slices through simulations containing little power on large scales (see Fig. 7 of ref. 10). It is difficult to understand, however, how so many features could maintain an organized regularity over such a long baseline. Also, it remains to be seen how the various theories can survive quantitative comparisons with our data.

Structures on scales of $\sim 100 \text{ Mpc}$ would be very difficult to detect without the extensive and deep redshift surveys that are only just beginning. If it were found that such structures are only prominent in some directions, this might be understood in terms of a cellular distribution of galaxies. Depending on the thickness of the shells, their regularity and spacing, and the distribution of galaxies within and on the cell walls, this might generate one-dimensional cross-sections whose form would strongly depend on direction. We present our results at this stage to underline the importance of pursuing more extensive deep galaxy and cluster redshift surveys to clarify these and other possible structures on scales of $\sim 100 h^{-1} \text{ Mpc}$ and above. □

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Amino-acid synthesis in carbonaceous meteorites by aqueous alteration of polycyclic aromatic hydrocarbons

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IT has been suggested that amino acids and other organic compounds found in carbonaceous meteorites formed by aqueous alteration in the meteorite parent bodies¹. Observations of carbonaceous material in interstellar grains and interplanetary dust particles^{2–4} indicate that condensed organic compounds may have been present in meteorite parent bodies at the time of aqueous alteration. One group of compounds thought to be representative of this carbonaceous material is the polycyclic aromatic hydrocarbons (PAHs)⁵. Recently it was proposed that PAHs condense on SiC grains in the molecular envelopes of carbon-rich red-giant stars⁶, which would allow for their subsequent incorporation into meteorite parent bodies during accretion. This incorporation mechanism is supported by the identification of SiC grains in carbonaceous chondrites⁷. The possibility therefore exists that PAHs, and/or other condensed organic compounds, represent the starting material for aqueous alteration which leads to the formation of amino acids and other water-soluble organic compounds. Here we present calculations of the distribution of aqueous organic compounds in metastable equilibrium with representative PAHs as functions of the fugacities of O_2 , CO_2 and NH_3 . The results reported here for pyrene and fluoranthene, two PAHs with different structures but the same stoichiometry, differ greatly but indicate that the formation of amino and carboxylic acids is energetically favourable at probable parent-body alteration conditions. The actual reaction mechanisms involved could be revealed by consideration of isotope data for PAHs, amino acids, other organic compounds and carbonates in carbonaceous chondrites^{8,9}.

Efforts have been made to explain the presence of amino acids in carbonaceous chondrites with Miller-Urey¹⁰, Fischer-Tropsch¹¹ and ion-molecule reactions¹² (see ref. 13 for detailed discussion). The Miller-Urey synthesis requires spark discharges in a vapour phase, the Fischer-Tropsch process involves formation of organic compounds from inorganic gases in reactions catalysed by mineral surfaces, and ion-molecule reactions involve the cosmic-ray ionization of species in interstellar clouds which react with other molecules at low temperatures (typically $\sim 10 \text{ K}$). If these vapour-phase and surface-catalysed reactions are the only possible sources of organic compounds, extensive

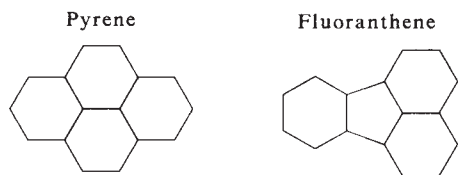
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reworking of minerals and carbonaceous material is required to explain the pervasive nature of organic compounds in carbonaceous meteorites. The presence of amino acids in meteorites having a phyllosilicate matrix has been taken to suggest that these solids, or the coexisting complex and partially polymeric organic matter, served as catalysts for reactions in which the amino acids formed¹. Alternatively, the amino acids could have been adsorbed onto the phyllosilicate and organic surfaces after formation. The spatial distribution of amino acids and other organic compounds, as well as their association with phyllosilicate minerals, can be explained by the formation of these organic compounds by the same aqueous-alteration events that formed the phyllosilicates. An example of the proposed makeup of the mineralogical and organic phases in a meteorite parent body during and after aqueous alteration is shown in Fig. 1.

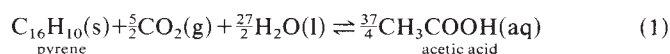
Evidence for aqueous alteration of the primary mineralogical composition of carbonaceous meteorites includes replacement textures, veins and the presence of carbonates, sulphates and hydrous silicates in the alteration assemblages¹⁴⁻¹⁸. It is generally believed that this alteration occurred on the parent bodies of the meteorites¹⁹. Suggested sources of water for alteration include ice^{20,21} and hydrated mineral phases²². The heat required to generate liquid water from these starting materials could have come from impacts²², release of accretionary kinetic energy²³, electromagnetic induction²⁴, or the decay of short-lived radionuclides^{1,21,25}. A partial course of aqueous alteration of meteoritic minerals has been tracked with electron microscopy¹⁸ and mass-balance calculations¹⁶. Oxygen isotopic ratios indicate that aqueous alteration occurred over a range of temperatures from ~20 °C on the Murchison parent body to ~140 °C on that of the Orgueil meteorite²⁶.

In contrast to the mineral assemblages, for which petrological analysis can differentiate primary minerals from aqueous-alteration products, methods are not established for identifying primary and secondary organic compounds in meteorites. Nevertheless, recent advances in theoretical geochemistry allow calculation of the thermodynamic properties of aqueous organic compounds over wide ranges of pressure and temperature²⁷, and permit new insights into geochemical processes in which organic matter and aqueous solutions interact. Studies of organic acids in sedimentary-basin brines^{28,29}, and the possible role of hydrothermal processes in the origin of amino and carboxylic acids during outgassing on the early Earth³⁰, demonstrate the importance of metastable redox equilibria among organic compounds in geochemical processes.

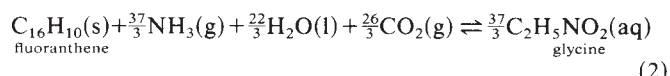
Simultaneous solution of mass-action and mass-balance equations allows calculation of the distribution of aqueous organic compounds. These calculations are designed to address the following questions: (1) what is the possible distribution of aqueous organic compounds at conditions under which solutions interact with PAHs on meteorite parent bodies? (2) what are the effects of differing activities of inorganic reactants on the metastable distribution of species? (3) are there differences in the results if different PAHs are considered as starting materials? In these calculations we have used pyrene and fluoranthene, two C₁₆H₁₀ PAHs found in the Murchison meteorite³¹. The carbon-bond structures of these two compounds are



carbonates in the alteration assemblage, and we suggest that trace amounts of NH₃ could have been incorporated along with water ice^{3,21}. We propose that in the metastable states considered, aqueous organic species form owing to kinetic barriers that prohibit the formation of the inorganic species graphite and methane. Similar constraints for graphite were placed on calculations of gas-phase equilibria in the pioneering work of Dayhoff *et al.*³², and the exclusion of methane is consistent with observations of metastable redox equilibria in sedimentary-basin brines^{28,29}. Examples of the types of reactions considered are:



and



where (g), (l), (s) and (aq) refer to the gas, liquid, solid and aqueous states. Equilibrium constants for reactions involving 76 aqueous alkanes, alkenes, alkynes, alkylbenzenes, ketones, alcohols, carboxylic acids, amines and amino acids were calculated with equations and data given in ref. 27, together with thermodynamic data for pyrene and fluoranthene^{33,34}. We set the initial activities of solid pyrene and fluoranthene to 10⁻² to acknowledge that these are only two of many possible components of a complex, condensed organic phase in the meteorite.

In our calculations we used 100 °C, 100 bar and an NH₃ fugacity, f_{NH_3} , of 10⁻³ to simulate conditions within meteorite

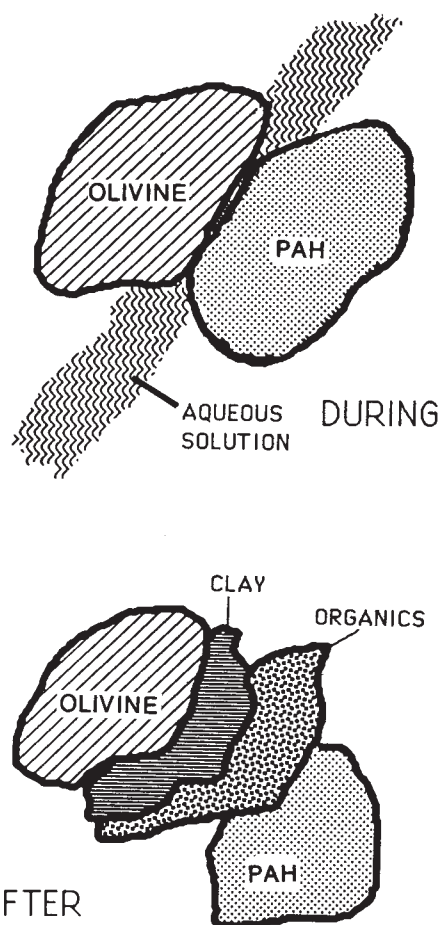


FIG. 1 Schematic diagram depicting simultaneous alteration of inorganic and organic carbonaceous chondrite material by aqueous solution. 'Clay' refers to phyllosilicate alteration products and 'PAH' designates polycyclic aromatic hydrocarbons.

In our calculations the C, N, H and O in starting assemblages of the solid PAH, H₂O, CO₂ and NH₃ are redistributed into metastable assemblages that include aqueous organic compounds. The choice of CO₂ is consistent with the occurrence of

parent bodies during alteration. Results for pyrene are shown in Fig. 2, which contains log-log plots of the activity of aqueous organic compounds, a_{organic} , against f_{CO_2} . These results are given in one figure so that the behaviour of the various groups of organic compounds may be compared. The solid curves in Fig. 2 indicate the log a of individual aqueous organic species,

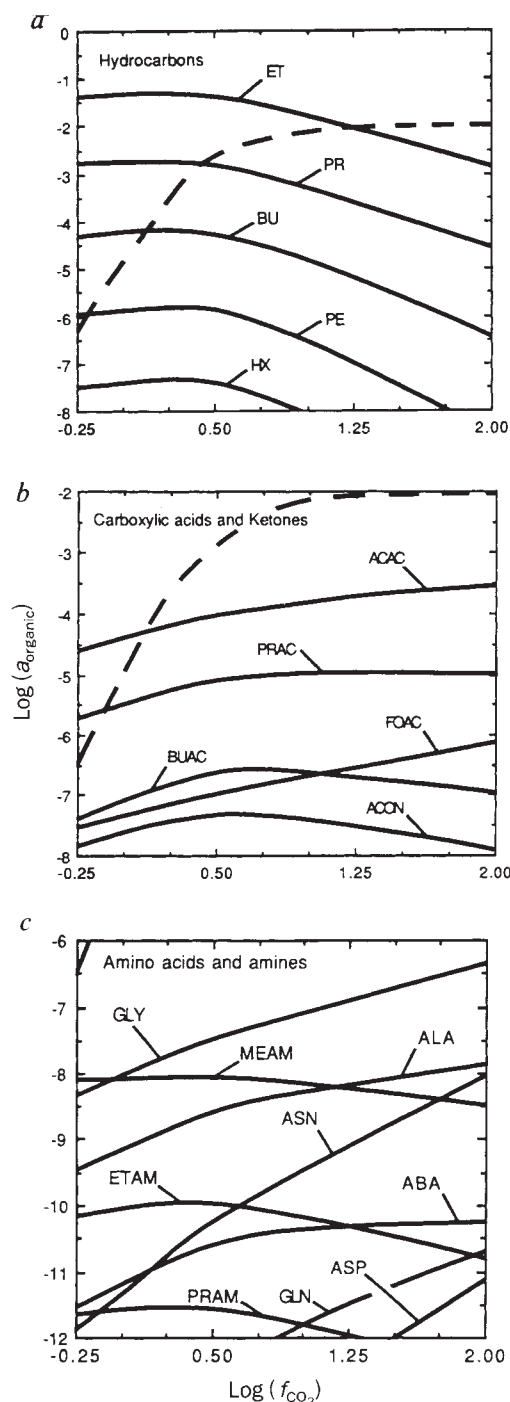
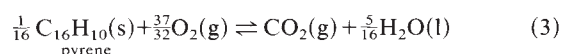


FIG. 2 Plots of the logarithmic activities of aqueous organic species as functions of $\log f_{\text{CO}_2}$ in metastable equilibrium with pyrene at 100 °C, 100 bar, and f_{NH_3} set to 0.001. *a*, Aqueous hydrocarbons (ET, ethane; PR, propane; BU, butane; PE, pentane; HX, hexane); *b*, aqueous carboxylic acids and ketones (ACAC, acetic acid; PRAC, propanoic acid; BUAC, butanoic acid; FOAC, formic acid; ACON, acetone); *c*, aqueous amino acids and amines (GLY, glycine; ALA, alanine; ASN, asparagine; GLN, glutamine; ABA, α -aminobutyric acid; ASP, aspartic acid; MEAM, methanamine; ETAM, ethanamine; PRAM, propanamine). In each plot, the dashed curve indicates the log a_{pyrene} at metastable equilibrium.

and the dashed curves correspond to the log a of solid pyrene at metastable equilibrium. The ordinate is truncated at -8 in Fig. 2*a*, *b*, indicating that log a values of many of the aqueous organic compounds considered in the calculations (such as alkylbenzenes, alkenes, alkynes) do not become this large. The ordinate in Fig. 2*c* extends to -12 to show the activities of the predominant nitrogen-bearing reaction products.

Figure 2*a* shows that the activities of aqueous hydrocarbons increase with decreasing f_{CO_2} , and that at these conditions pyrene is reduced to an aqueous assemblage dominated by simple alkanes. By contrast, the activities of acetic, propanoic and formic acids (Fig. 2*b*) as well as those of glycine, alanine, α -aminobutyric acid, asparagine, glutamine and aspartic acid (Fig. 2*c*) all increase with increasing f_{CO_2} . We conclude that organic acids are the energetically favoured reaction products of aqueous alteration of pyrene at high values of f_{CO_2} . The fugacity of CO_2 can be related to the oxygen fugacity of the system by the reaction:



for which we calculate a log K of 67.8 at 100 °C and 100 bar. Using this equilibrium constant, values of $\log f_{\text{CO}_2}$ can be calculated from values of $\log f_{\text{O}_2}$ and $\log a_{\text{H}_2\text{O}}$. We assume that an aqueous solution of this type would have been dilute, and that the activity of H_2O was close to unity.

It is likely that the fugacities of oxygen imposed on reaction (3) would be buffered by mineral assemblages in a meteorite parent body. At present we cannot calculate unequivocally the values of f_{O_2} that prevailed during the alteration event because of the lack of thermodynamic data for many of the iron-, nickel- and sulphur-bearing phases in the alteration assemblage (Fe-serpentines, tochilinites, Fe-chlorites, for example). Nevertheless, the results of two theoretical studies of aqueous alteration in parent bodies^{25,35} are consistent with oxygen fugacities at or near those set by the nickel-bunsenite assemblage.

Using the value of $\log f_{\text{O}_2}$ (-57.3) for this assemblage at 100 °C and 100 bar, together with the equilibrium constant for reaction (3), yields $\log f_{\text{CO}_2} = 1.4$ at equilibrium with pyrene and water (with activities of 10^{-2} and 1, respectively). We used these values, together with $f_{\text{NH}_3} = 10^{-3}$, to calculate the activities of aqueous organic species at metastable equilibrium listed in Table 1. The results for similar calculations for reactions using fluoranthene as the starting material are listed in Table 2. The calculated equilibrium constant for the fluoranthene analogue of reaction 3 at 100 °C and 100 bar is 68.4, which yields $\log f_{\text{CO}_2} \approx 2.0$, which is consistent with the nickel-bunsenite assemblage.

Comparing the possible products of aqueous alteration from these two $\text{C}_{16}\text{H}_{10}$ PAHs, we note that in both cases, the log a

TABLE 1 Logarithmic activities of aqueous organic species using pyrene as starting material

Species	log a	Species	log a
1. Ethane	-2.20	14. Ethanol	-8.51
2. Acetic acid	-3.68	15. Toluene	-8.60
3. Propane	-3.81	16. Methanol	-8.66
4. Propanoic acid	-4.95	17. Asparagine	-8.85
5. Butane	-5.47	18. Hexane	-9.03
6. Formic acid	-6.45	19. 2-butanone	-9.14
7. Butanoic acid	-6.73	20. Benzene	-9.20
8. Glycine	-6.77	21. Propanol	-9.87
9. Pentane	-7.30	22. Hexanoic acid	-10.09
10. Acetone	-7.60	23. α -aminobutyric acid	-10.31
11. Alanine	-8.09		
12. Methanamine	-8.27	24. Ethanamine	-10.41
13. Pentanoic acid	-8.36	25. Ethylbenzene	-10.41

Log $a_{\text{organic}} > -11$ are listed for aqueous species in metastable equilibrium with pyrene at 100 °C and 100 bar, $\log f_{\text{NH}_3} = -3$, $\log f_{\text{O}_2}$ set by the nickel-bunsenite assemblage.

TABLE 2 Logarithmic activities of aqueous organic species using fluoranthene as starting material

Species	log <i>a</i>	Species	log <i>a</i>
1. Ethane	-1.33	18. Propylbenzene	-7.72
2. Propane	-2.51	19. Heptane	-8.04
3. Acetic acid	-2.69	20. Methanol	-8.21
4. Propanoic acid	-3.51	21. Heptanoic acid	-8.46
5. Butane	-3.71	22. Propanol	-8.52
6. Butanoic acid	-4.85	23. 2-pentanone	-8.72
7. Toluene	-5.32	24. Butylbenzene	-8.89
8. Pentane	-5.16	25. Octane	-9.03
9. Formic acid	-5.91	26. Octanoic acid	-9.51
10. Pentanoic acid	-6.03	27. Phenol	-9.56
11. Acetone	-6.21	28. 1-propene	-9.80
12. Benzene	-6.37	29. Methanamine	-9.81
13. Hexane	-6.39	30. 2-hexanone	-9.85
14. Ethylbenzene	-6.68	31. Glycine	-10.27
15. 2-butanone	-7.30	32. Alanine	-10.37
16. Hexanoic acid	-7.30	33. Ethanamine	-10.74
17. Ethanol	-7.61		

Log $a_{\text{organic}} > -11$ are listed for aqueous species in metastable equilibrium with fluoranthene at 100 °C and 100 bar, log $f_{\text{NH}_3} = -3$, log f_{O_2} set by the nickel-bunsenite assemblage.

values of the carboxylic acids are among the largest, but their activities are roughly one order of magnitude higher in the fluoranthene assemblage. By contrast, amino acids have higher activities in the pyrene assemblage. As an example, glycine ranks eighth in the assemblage in metastable equilibrium with pyrene, and thirty-first in the fluoranthene assemblage. In addition, the activities of many alkanes and alkylbenzenes are higher in the fluoranthene assemblage than in the pyrene assemblage. This comparison indicates that widely differing assemblages of aqueous organic species are possible from PAHs with the same stoichiometry.

These results may help to explain the isotopic composition of various organic compounds in carbonaceous meteorites. For example, amino acids extracted from the Murchison meteorite^{9,36} have a $\delta^{13}\text{C}$ ($= [({}^{13}\text{C}/{}^{12}\text{C})_{\text{sample}}/({}^{13}\text{C}/{}^{12}\text{C})_{\text{standard}}] - 1$) range of +23 to +44‰, $\delta\text{D} = +1,370$ ‰ and $\delta^{15}\text{N} = +90$ ‰, but the fraction of condensed organic matter identified as pre-

dominantly aromatic⁸ has $\delta^{13}\text{C} = -18.7$ ‰, $\delta\text{D} > 1,300$ ‰ and $\delta^{15}\text{N} < -6$ ‰. Therefore, alteration of PAHs to produce amino acids requires isotopically heavy carbon and nitrogen in the aqueous phase. This seems to be consistent with the CO_2 ($\delta^{13}\text{C} = +29.1$ ‰)³⁷ and carbonates ($\delta^{13}\text{C} = +44$ to $+60$ ‰)^{36,38} extracted from the Murchison meteorite, as well as the nitrogen in low-temperature methanol extracts ($\delta^{15}\text{N} = +90$ ‰)³⁹. Trends in more-detailed carbon isotope data for light hydrocarbons and carboxylic acids from the Murchison meteorite³⁷ may reflect metastable equilibria among these compounds, in which the average oxidation state of carbon varies, and redox reactions involving condensed organic precursors and an isotopically heavy aqueous phase. These trends can be used to elucidate the paragenesis of metastable organic compounds during aqueous-alteration events, as well as the kinetically favoured pathways. Recent observations of the isotopic composition of PAHs from the Murchison meteorite (I. Gilmour, personal communication) will allow quantitative tests of this aqueous-alteration hypothesis.

Our results demonstrate the possibility that reactions involving aqueous solutions and PAHs could have produced assemblages of aqueous organic species during the alteration events documented in the mineralogical composition of carbonaceous chondrites. In this regard, the recently reported spatial distribution of PAHs and their apparent association with matrix minerals in the Allende meteorite⁴⁰ is strikingly similar to that in the proposed alteration scenario. Our calculations also reveal the controlling influence of f_{O_2} , f_{CO_2} and f_{NH_3} on the resulting metastable assemblages of organic compounds. It should be noted that we have used a low fugacity of NH_3 , and that the activities of the amino acids would increase dramatically at higher ammonia fugacities. These are calculations for only two of the many possible condensed organic starting compounds. The differences observed between these two similar compounds suggest that vastly different results will be found for other PAHs, heterocyclic compounds, paraffins and other possible reactants. Once the consequences of metastable equilibria have been established for a wide variety of likely starting materials, isotope evidence from individual organic compounds can be used to constrain the relative abundances of condensed organic compounds in the assemblages before aqueous alteration, together with likely reaction pathways, in mass-transfer calculations for coupled inorganic and organic species. □

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The mass of spinning rotors: no dependence on speed or sense of rotation

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ANOMALOUS mass reductions in spinning gyroscopes have been reported by Hayasaka and Takeuchi¹. For one sense of rotation about a vertical axis the mass of the gyroscope appeared to be a linear function of the speed of rotation (albeit with non-zero intercept); in the other direction it did not. In the absence of any theoretical explanation of such behaviour these results are very puzzling. Here we present the results of weighing a 330-g spinning rotor as it freely spins down from speeds of 8,000 r.p.m. We observe some changes in its apparent mass, which are a function of both speed and sense of rotation, but they amount to only ~5% of what would be required for consistency with ref. 1. If corrections are made for the effects of the friction couple slowing the rotor, and for changes in temperature, the observed effect is reduced by more than a factor of ten, and is no longer significant.

Although well equipped with balances, we had no experience in gyroscopes and thus our initial reaction on reading ref. 1 was to leave the problem for others, better placed than ourselves, to solve. But on 18 January 1990, J. Faller from the Joint Institute for Laboratory Astrophysics in Boulder, Colorado gave a talk at our institute in which he described weighings of an elegant and simple gyroscope. His results² did not show the anomalous behaviour reported in ref. 1, and encouraged by this experiment, we decided to carry out a similar project.

The rotor is shown in Fig. 1 and closely follows the design shown to us by Faller. It is made from a cylinder of brass ~40 mm in diameter and 30 mm high, supported on jewel bearings by tungsten points. Four turbine blades were machined into the top of the rotor to allow spin-up by one of two pairs of gas jets. The direction of rotation was decided by the choice of jets. In the absence of equipment for dynamically balancing the rotor, we simply employed careful machining and measurement. As in the rotor used by Faller, the jewel bearings were located in spring mounts to allow some movement. The rotor was fitted inside a cylindrical enclosure made from perspex, the whole assembly having a mass of ~900 g. The thick perspex walls allowed the spinning rotor to be seen, and its speed to be measured using a stroboscope. They also contributed some thermal insulation, so that any frictional heating of the gas would be slow to raise the temperature of the external surfaces. In the event, we concluded that at the speeds we used, most of the frictional losses were in the bearings and not in the gas. The decay time of the rotor from 10,000 r.p.m. to rest increased only from ~8 to 10 minutes when helium instead of air was used to drive it. For the measurements presented here we used air to drive the rotor. The laboratory compressed-air supply is slightly damp, which might help to avoid the build-up of electrostatic charge—a serious problem in weighing.

For this experiment we used the flexure-strip balance developed in our laboratory^{3,4}, which has very high sensitivity and has already been used in an experiment to search for non-newtonian gravity^{5,6}. In that 'fifth-force' experiment, masses of ~2.3 kg were compared with a standard deviation of 6 ng, a relative uncertainty of 2.6 parts in 10^{12} . We did not expect to be able to measure the mass of a spinning rotor to anything like this precision, but we were confident that we had a good understanding of the behaviour of the balance.

If the results of ref. 1 are not evidence of new physics, the source of the anomalous behaviour is likely to be found in the way that the balances used interacted with the gyroscope. In

our flexure-strip balance, special care was taken in the design of the pan suspension. Ideally, a pan should be suspended from the end of the beam by a perfect, frictionless gimbal. If not, any tilt of the pan, whatever its cause, is translated into a couple acting on the beam which cannot be distinguished from a change in mass. In our balance the novel gimbal system reduces, by a factor of at least 10^5 , any effect resulting from tilt of the pan³. The beam should also be completely insensitive to couples about a vertical axis; in practice, this is impossible to achieve and, as shown below, such couples can lead to anomalous mass measurements. The effects of both tilt and vertical couples are intensified if the flexures or, in the case of a knife-edge balance, the knives are not parallel.

Because the flexure-strip balance was designed for 2-kg masses, we added to each pan a 1-kg pan plate made from lead and brass in order to increase the mass of the total weighed object and thus reduce effects of vibration. The tare mass, in addition to the extra pan plate, was made from 330 g of brass and 570 g of aluminium alloy. In this way the tare and test masses were approximately balanced for buoyancy effects.

The experiment was carried out as follows. The rotor was spun up to 14,000 r.p.m. by compressed air, the air jets were

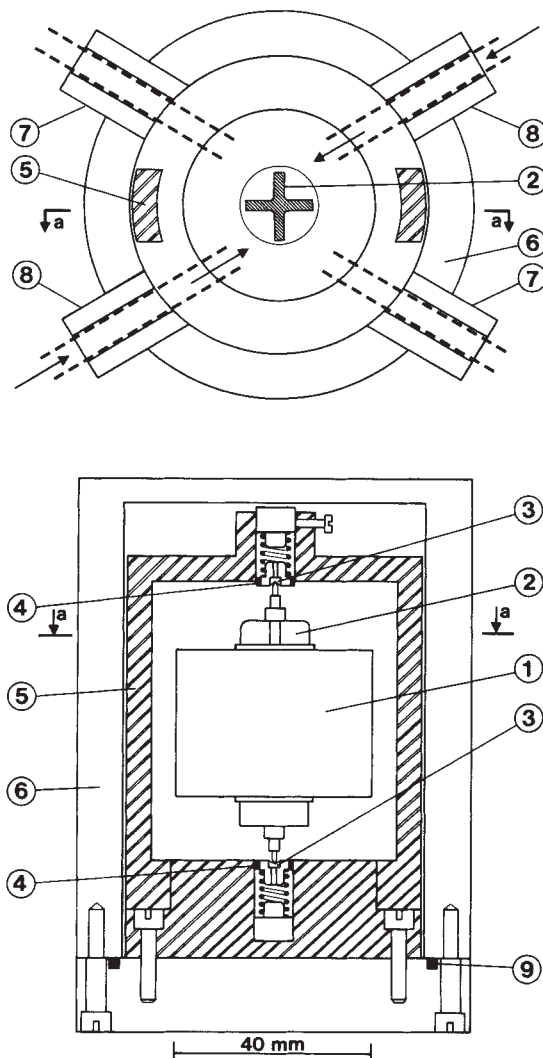


FIG. 1 The spinning rotor assembly: 1, brass rotor; 2, turbine blades; 3, spring-loaded jewel bearings; 4, rubber bushing; 5, aluminium-alloy support for bearings; 6, perspex case; 7, guides for gas-drive injector tubes (clockwise rotation); 8, guides for gas-drive injector tubes (anticlockwise rotation); 9, "O" ring.

removed and small rubber plugs inserted into the ports. The assembly was then placed, by hand, on the pan of the balance, the servo system was engaged and the balance case was closed to provide a hermetically sealed enclosure. By the time the initial disturbance had subsided and the servo system of the balance had taken control, the speed had decreased to $\sim 9,000$ r.p.m.. From this point on, until some time after the rotor had stopped spinning, both the servo current, I , and the air temperature, T , measured just above the rotor assembly, were recorded.

A curve of spin rate, S , as a function of time had previously been established and was found to be very reproducible. The speed decreased more rapidly at the outset, and fell to zero with a finite slope. During the measurements described here the speed

at any instant could be deduced using this calibration curve. For purposes of comparison, the time at which the rotor stopped spinning was taken as the time origin. This was always clearly visible on the recorder trace, for reasons we explain below. The experiment was repeated several times with the rotor spinning in each direction, referred to as clockwise or anticlockwise viewed from above, and for a range of initial spin rates from 14,000 to 7,000 r.p.m.. A typical example of what we observed is shown in Fig. 2*a*, curve A. As the speed of rotation decreased, several resonances were clearly visible. These were very reproducible and proved useful as markers, but were otherwise of no importance.

From the results of ref. 1, we would predict a change in

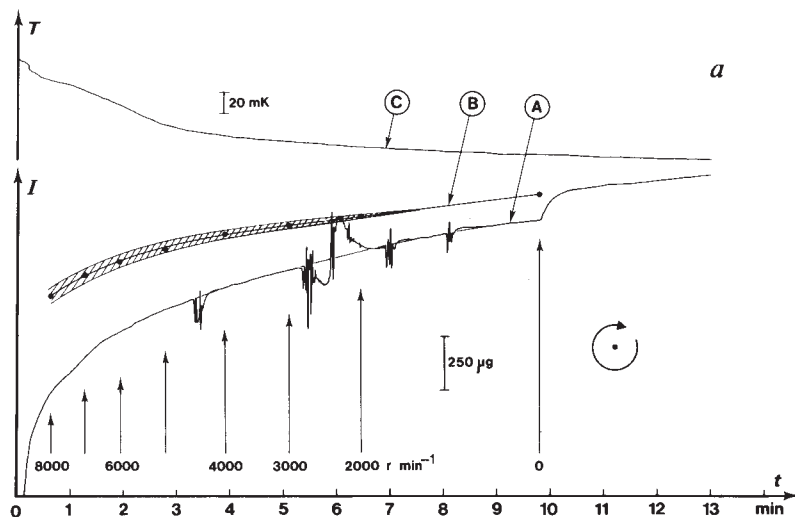


FIG. 2 *a*, Clockwise rotation of the rotor: A, servo current I as a function of time during the spin-down of the rotor; C, air temperature T measured just above the rotor assembly as a function of time; B, smoothed trace A after correction for effects of the friction couple; the hatched area either side of the corrected curve B shows our estimate of the 1σ uncertainty of the correction. The jump in I at time $t \approx 9.8$ min is due to the stopping of the rotor. A positive slope in $I(t)$ indicates an apparent mass increase. *b*, Anticlockwise rotation of the rotor. At $t \approx 10.7$ min the servo current falls as the rotor stops. *c*, The servo-current curves from *a* and *b* superposed so that they coincide during the period when the rotor is stationary.

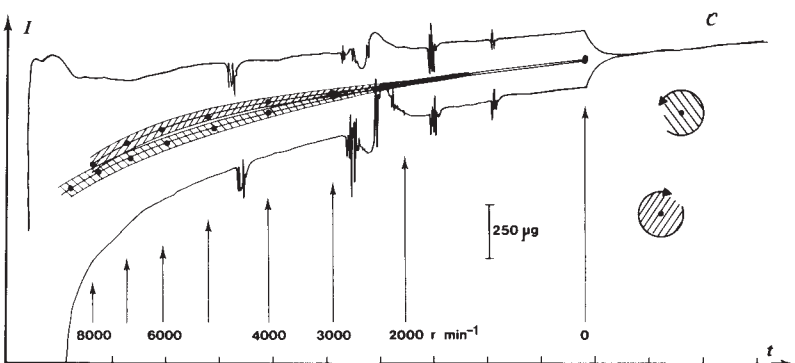
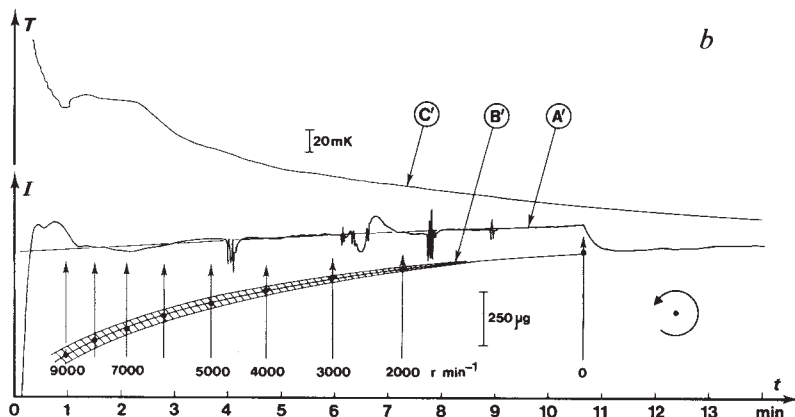
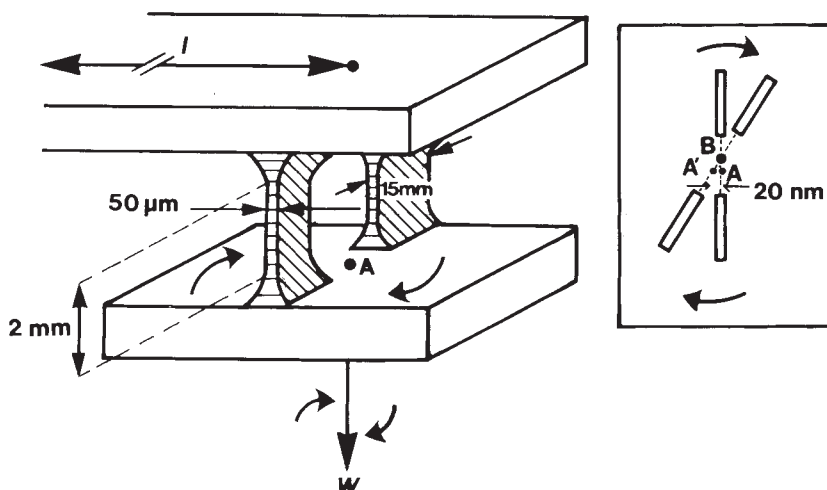


FIG. 3 Illustration (not to scale) of the effect of the friction couple on apparent mass. The pans are suspended from two thin flexure strips (hatched). The friction couple is applied about a vertical axis through A, the effective point of application of the weight. If this couple leads to a true rotation of the flexure strips about A there will be no apparent change in mass, because the effective arm length of the balance, l , remains unchanged. But if, as is more likely, because of small differences in stiffness between the two strips, the rotation is about another point slightly displaced from A (point B in the top view on the right), the effective point of application will change (to A') and so, therefore, will l . The couple will thus lead to an apparent change of mass. A displacement of ~ 20 nm is sufficient to cause the observed change in mass; therefore it is not surprising that the effect is of different magnitude at each of the ends of the beam. It is also clear that rotation in opposite directions causes the small displacement to occur in opposite directions. In general, any couple applied to a balance about any axis will appear as a change in apparent mass. Although the constant of proportionality may be small, it is always finite.



apparent mass, for one sense of rotation only, of ~ 13.6 mg between a stationary rotor and one spinning at 8,000 r.p.m.. From a cursory examination of Fig. 2, it is evident that if any effect of the sort reported in ref. 1 is present in our measurements, it is only at the level of a few tenths of a milligram—that is, only a few per cent of what these results would imply for our rotor. This immediately confirms the conclusion of Faller *et al.*², who set an upper limit of $\sim 4\%$ on Hayasaka and Takeuchi's prediction.

A closer examination of Fig. 2, however, seems to show an effect similar to that seen by Hayasaka and Takeuchi¹. The mass of the rotor spinning in the clockwise direction seems to depend significantly on the rotation speed, but in the anticlockwise direction the effect is much smaller. We will show that this effect can be attributed to the sensitivity of the balance to couples acting about a vertical axis, combined with the effect of temperature variations of the rotor assembly.

The temperature of the rotor assembly begins to decrease soon after it is placed on the pan of the balance, having previously been raised a little during handling and rotor spin-up. The kinetic energy of rotation, less than 100 J, is insufficient to reverse this trend. The decrease in temperature of the rotor assembly leads to an increase in its apparent mass, as convection is by far the largest temperature-dependent parameter affecting the measurement of its mass. No quantitative correction could be made for temperature drift as it was too difficult to measure the temperature of the rotor assembly while it was being weighed. The rate of drift of apparent mass, however, was correctly correlated in sign and approximate rate with the change in air temperature measured just above the rotor, where it acts as an indicator of convection (Fig. 2, curves C, C'). It is important to note that the temperature drift during spin-down of the rotor was always in the same direction, independent of the sense of rotation of the rotor.

We come now to the interaction of the beam of the balance with the rotor spinning on the pan. The spinning rotor slows by the action of friction couples, applied mostly through the bearings. These couples, which act about a vertical axis, are passed on to the pan and thence, by means of the pan suspension (the flexure strips; see Fig. 3) to the end of the beam. The presence of these couples and the effect on the beam are shown quite unequivocally by the change that takes place in the servo current at the moment the rotor stops rotating. The friction couple falls sharply to zero, and its sign and magnitude are thus revealed on the trace (points marked "0" in Fig. 2). As the

friction couple is proportional to $\dot{\omega}$, that is, to dS/dt , a knowledge of its effect on apparent mass at one point, together with values of $\dot{\omega}$ read from our curve of S as a function of time, allows us to calculate the effect on apparent mass for all values of S . The couple acts, of course, in opposite directions for the two senses of rotation, and the apparent mass corrections are thus positive in one case and negative in the other.

If we now apply the friction-couple correction to each trace we should be left with the variation in apparent mass due to temperature drift or to some other, unknown, physical effect. The temperature drift after the rotor has stopped spinning is shown as the continuation of each servo-current trace in Fig. 2. By superposing the corrected traces we find that the servo currents before and after the rotor stops spinning are continuous, and the difference between clockwise and anticlockwise traces is now zero up to 2,000 r.p.m., increases to $68 \mu\text{g}$ at 6,000 r.p.m., and then decreases to $63 \mu\text{g}$ at 8,000 r.p.m.. Our estimated uncertainty (1σ) of the friction-couple correction is shown by the hatched area surrounding each corrected curve, and is $\sim 35 \mu\text{g}$ at 8,000 r.p.m.. We think that these remaining differences, which in any case are hardly significant, between the apparent mass for clockwise and anticlockwise rotations are suggestive of a variation in temperature between runs, but we are unable to make a quantitative estimate of the effect. The curvature that is now common to the corrected clockwise and anticlockwise traces is in the direction we would expect if it were due to the decreasing temperature of the rotor assembly. We find no sign of any effect that might be due to precession of the gyroscope resulting from the rotation of the Earth, amounting to nearly three degrees during the spin-down of the rotor. To test the absence of precession effects, we displaced the pan slightly while the rotor was spinning at $\sim 8,000$ r.p.m.. A precession could be induced, with a period of ~ 1 s, which could be seen on the servo-current trace, but it died away very quickly.

Our limit of $\sim 60 \mu\text{g}$ on the change in mass of our rotor as a function of spin direction is equivalent to 2 parts in 10^7 of its mass, or $\sim 0.4\%$ of what would be required for consistency with the results reported in ref. 1. We have also carried out two further experiments. In the first, we exchanged the spinning rotor with the tare mass on the other pan of the balance. We found an effect of the same sign (that is, a rotor spinning clockwise appears to increase in mass as it slows), but twice as large. In the second, we tightened the bearings of the rotor so that it slowed down much more quickly. This increased the observed effect by a further factor of 2.5. We take these

observations as confirmation that what we have observed is indeed an artefact of the balance, and not a new physical phenomenon. □

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Observation by neutron diffraction of the magnetic flux lattice in single-crystal $\text{YBa}_2\text{Cu}_3\text{O}_{7-\delta}$

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THE high-transition-temperature (high- T_c) oxide superconductors are type 2 superconductors: in an applied magnetic field, magnetic flux can enter the superconductor as quantized flux lines. In conventional type 2 superconductors, these lines form a lattice¹, but in the new materials the flux lattice seems to be appreciably influenced by thermal fluctuations and may ‘melt’ at temperatures well below T_c (refs 2–4). Flux lines in high- T_c materials have been imaged by ‘decorating’ the ends of the lines with tiny magnetic particles^{5,6}, but such experiments are limited to very low fields and indicate only the behaviour at surfaces. Here we report the observation of magnetic flux lines in the bulk of a crystal of $\text{YBa}_2\text{Cu}_3\text{O}_{7-\delta}$, using small-angle neutron diffraction. The experimental data at low temperatures confirm the existence of a vortex lattice of singly quantized flux lines. The results at higher temperatures show that neutron diffraction can be used to investigate the melting of the flux lattice.

When a type 2 superconductor is in the mixed state (in which flux penetrates the superconductor), the local magnetic field has a maximum value at the flux-line cores and a minimum in the regions between them. The mixed state within the bulk can therefore be probed by any microscopic technique that is sensitive to this field contrast. For instance, muon spin resonance (μSR) experiments can supply information on the extent of the field non-uniformity in the mixed state⁷. Such investigations have been successful in determining the temperature dependence of λ_L , the London penetration depth^{8,9}, but can provide only indirect information on the structure of the flux lattice itself. Another microscopic technique is the diffraction of neutrons by the flux-line structure in the mixed state. This technique has been used widely to study conventional type 2 superconductors^{1,10} ranging from niobium to technetium. This method has not, however, yet provided any information for

oxide superconductors. We and others have tried previously and failed to detect any scattering from the flux lattice. One reason why such experiments have so far been unsuccessful is that the diffraction of neutrons relies on the magnitude of the field contrast in the mixed state; this contrast is very small for high- T_c materials, all of which have large London penetration depths. If the flux lines form a lattice, the integrated scattered intensity I_{hk} for the (h, k) Bragg reflection may be written as¹¹:

$$I_{hk} \propto \frac{|F_{hk}|^2}{\sin \theta_{hk}} \quad (1)$$

where θ_{hk} is the Bragg angle for the (h, k) reflection from the lattice and F is the ‘form factor’, which is proportional to the magnitude of the field contrast. In extreme type 2 materials, such as the high- T_c superconductors, the effect of the flux-line cores can be neglected unless the flux density B is near to the upper critical flux density B_{c2} , which would apply only close to T_c . Under such circumstances the solution of the London equations leads to an explicit expression for F :

$$F_{hk} = \frac{B}{1 + (2\pi\lambda_L/d_{hk})^2} \quad (2)$$

where d_{hk} is the spacing between the (h, k) planes. The $(1, 0)$ reflection, with the largest d -spacing, will have the greatest intensity, and if $d_{10} < \lambda_L$, which implies that $B > B_{c1}$ (the lower critical flux density), the second term in the denominator of equation (2) dominates, so that

$$I_{10} \propto d_{10}^2 \frac{\Phi_0^2}{\lambda_L^4} \quad (3)$$

where Φ_0 is the flux quantum $= h/2e$.

Thus, for a given flux-line spacing, the scattered intensity would be greatly reduced in a material with a long penetration depth. Unfortunately, in high- T_c materials, the low-temperature values of the penetration depths are greater than that of Nb by factors of at least four, and in some cases by much more.

The angle of diffraction depends on the flux-line lattice d -spacing. This is controlled by the flux density B within the specimen, because there will be one flux quantum per unit cell of this lattice. For a hexagonal flux lattice,

$$d = \sqrt{\frac{\sqrt{3}}{2}} \sqrt{\frac{\Phi_0}{B}} \quad (4)$$

With an applied magnetic field of 0.2 T this expression predicts $d \approx 1,000 \text{ \AA}$, which implies very-small-angle scattering; this would have to be detected in the presence of additional scattering from twins, cracks or other inhomogeneities which are prevalent in high- T_c crystals.

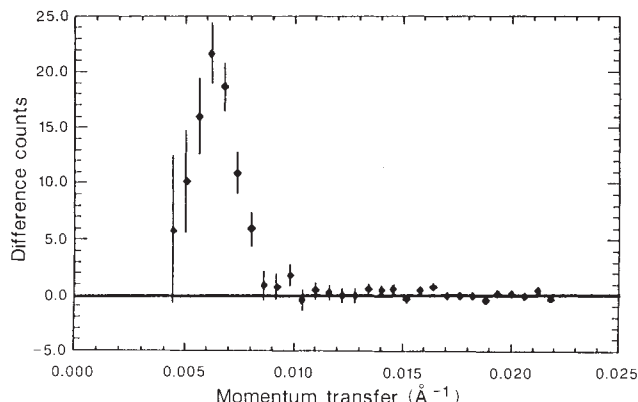


FIG. 1 The extra neutron scattering observed with the sample in the mixed state at 20 K and 0.2 T. The neutron counts are tangentially averaged around the beam axis. The horizontal coordinate is a measure of the scattering angle.

In these experiments we used the neutron small-angle-scattering instrument D11 of the Institut Laue-Langevin¹², with a neutron velocity selector of 9% full width at half maximum, to produce a flux of neutrons with mean wavelength of 10 Å. The incident neutrons were collimated over a distance of 10 m before reaching the sample. A 64×64-cm multidetector at distances between 10 and 15 m was used to detect the scattered neutrons. The sample was mounted in a cryostat in a magnetic field of 0–0.2 T, applied parallel to the incident beam. In this geometry, analogous to that employed for diffraction in a transmission electron microscope, the scattering vectors of the lattice are perpendicular to the beam. The scattering angles are so small ($\leq 0.5^\circ$ in these experiments) that a flux-line lattice mosaic spread of the same order will bring the lattice planes to the correct orientation to satisfy the Bragg condition. Thus the plane of the detector represents a plane of reciprocal space of the flux-line lattice. Even if the lattice is completely random in orientation about the field axis, there should still be a ring of scattered intensity around the transmitted beam.

The sample was a 150-mg single crystal of $\text{YBa}_2\text{Cu}_3\text{O}_{7-\delta}$ grown by the halide flux method. It was annealed in flowing oxygen, being cooled from 500 to 150 °C over 350 hours. Measurements of a.c. susceptibility indicate that the crystal had a slightly split transition with onsets at 93 K and 85 K. The crystal was in the form of a plate ~1 mm thick along the *c* axis, which was aligned parallel to the magnetic field within a few degrees.

In the normal state, the 'background' scattering from the sample was independent of magnetic field and, in zero field, was independent of temperature. When the sample was cooled in a magnetic field, however, a small amount of additional scattering was observed. This additional intensity could be observed clearly by subtracting a background measurement in zero field from a field-cooled one. Figure 1 shows a tangential average of this additional scattering at 20 K as a function of the scattered momentum transfer (*q*). The position of the peak is consistent with trapping of all the applied magnetic field as singly quantized flux lines in the mixed state on cooling below T_c . An additional test of the origin of this peak was made by re-growing the flux-line lattice in a lower magnetic field; as

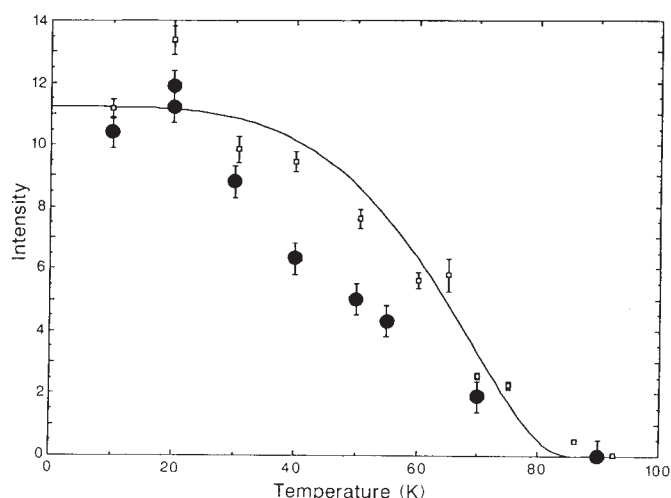


FIG. 3 Temperature dependence of flux-line-lattice scattering. Solid line: predictions of equation (6); full circles: intensity of scattered neutrons, measured by peak height of tangentially averaged data (as in Fig. 1); open squares: μSR relaxation rate squared, measured at the Paul Scherrer Institut on the same sample. This should have the same temperature dependence as the neutron scattering if the flux-line arrangement is independent of temperature.

expected, the peak shifted to lower momentum transfers and increased slightly in intensity. It is clear from Fig. 1 that errors in the subtractions become rapidly larger at small values of momentum transfer: this is due to the large increase in the background scattering, which was found to vary as $\sim q^{-3}$ at small *q*.

In Fig. 2 we show a contour plot of the neutron counts obtained from a total of 4 hours counting at a temperature of 20 K, the lattice being prepared by cooling the crystal through T_c in an applied field of 0.2 T, after the subtraction of a background of similar statistical significance. It is evident that the scattering from the flux lines does not form a featureless ring: Bragg spots and additional local structure can be detected in positions that are aligned with the twinned crystalline axes with an average fourfold symmetry. This does not imply that the flux-line lattice is itself square: it is likely that different regions of the crystal contain flux lattices in different orientations, and that the observed pattern is the sum of the patterns from each. It is possible, for instance, that the local orientation of the flux lattice is linked to twin planes.

Finally we consider the temperature dependence of the scattering from the flux-line lattice of $\text{YBa}_2\text{Cu}_3\text{O}_{7-\delta}$. Equation (3) shows that this depends on the variation of λ_L , but there may be additional effects if the flux lattice melts. μSR measurements^{8,9} in the mixed state show that the temperature dependence of λ_L is similar to the 'two-fluid' empirical expression:

$$\frac{\lambda_L(T)}{\lambda_L(0)} = \frac{1}{[1 - (T/T_c)^4]^{1/2}} \quad (5)$$

For our experiments at constant (trapped) magnetic induction, equations (3) and (5) give:

$$I \propto \left(1 - \left(\frac{T}{T_c}\right)^4\right)^2 \quad (6)$$

For the data presented in Fig. 3 a fit to the above expression, with T_c as an adjustable parameter, suggests that the effective T_c is ~67 K, but the quality of the fit is poor. In Fig. 3 the model curve is not a fit (the scaling factor was estimated from the average of the data for 10–20 K, and T_c was fixed at 85 K) but is simply an indication of the predictions of equation (6) with the minimum value of T_c that we consider to be appropriate. An investigation of the μSR relaxation rate (σ) for the crystal

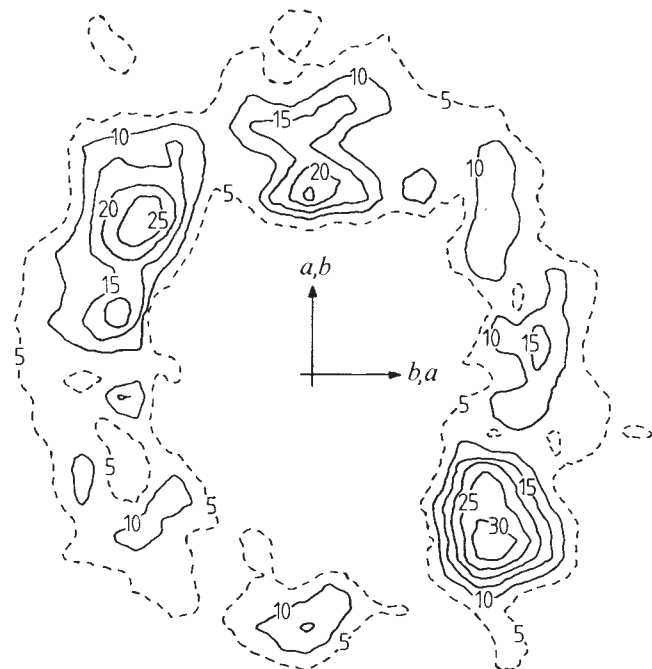


FIG. 2 Contour plot of flux-line-lattice scattering: difference at 20 K between field-cooled and zero-field data as a function of position on the multidetector. The arrows indicate the directions of the twinned (*a, b*) crystal axes.

used in this experiment suggests that the low-temperature value of λ_L is larger than that expected for a fully oxygenated sample and hence there is probably a degree of inhomogeneity in the oxygen stoichiometry within the bulk of the crystal. However, the temperature dependence of σ^2 , which should be proportional to F^2 for neutron scattering, can be scaled to lie close to the model curve in Fig. 3. Thus there is an intriguing difference between the neutron data and the model curve, but it would be premature to take this as a definite sign of the melting of the flux-line lattice. Nonetheless these experiments clearly demonstrate the potential of neutron scattering as a tool for investigating the local and long-range structure of the magnetic field in the mixed state for high- T_c superconductors. $\square$

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Femtosecond laser observations of molecular vibration and rotation

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ULTRAFAST molecular vibrations and rotations are the fundamental motions that characterize chemical bonding and determine reaction dynamics at the molecular level. The timescales for these motions are typically 10^{-13} s for vibrations and 10^{-10} s for rotations. For decades, time-integrated (frequency-resolved) spectroscopy has provided a powerful tool for probing the dynamics of motion, but the motions themselves are not 'seen' directly in real-time. With femtosecond laser techniques^{1–4} it is now possible to follow the motions of isolated molecular systems as they occur. The requirement is that the system is excited (for vibration) and aligned (for rotation) on a timescale shorter than the vibrational and rotational periods. Here we report real-time observations of these molecular motions. The system—in this case, molecular iodine—is prepared in the particular state(s) of interest by coherent excitation with an initial femtosecond laser pulse, and the subsequent motions are probed with successive femtosecond pulses. The probe monitors changes in the interatomic distance (vibration) or molecular orientation (rotation), so that the measured signal provides direct 'snapshots' of the molecular motions.

Figure 1 illustrates the fundamental motions for simple vibrating (harmonic) and rotating diatomic molecules. The vibrational motion (Fig. 1a) describes the change in interatomic distance, R , which is characterized by the natural frequency, ν , of the oscillator. The real-time rotational motion of a molecular ensemble (Fig. 1b) can be understood by considering the following simple motion of, say, three rotors. At time $t=0$, the rotors are aligned in the same direction. As time passes, the molecules

rotate at different angular velocities (depending on the angular-momentum quantum number $J=1, 2, 3$) and the initial alignment is lost. At $t=1/(8B)$ and $3/(8B)$ (where B is the rotational constant of the diatomics), the degree of alignment of the molecules is at a minimum. At $t=1/(2B)$, all the molecules have performed an integral number of rotations, so that there is a recurrence of the alignment. Thus the full rotational period of the motion is $1/(2B)$. To observe these motions in real-time, the probe must be sensitive to changes in R (for vibration) and in alignment (for rotation).

Femtosecond transition-state spectroscopy (FTS) is our tool for these experiments. In previous FTS studies^{5–8} of chemical reactions, the transition-state region between reagents and products was probed. When studying molecular motions of a bound state, there exists, of course, no transition state in the conventional sense; but one is still monitoring transitory changes of R and alignment, so that FTS can be generalized to investigate bound as well as unbound states. The technique uses two femtosecond pulses of different wavelengths and known relative polarizations. The first ('pump') pulse (λ_1 in Fig. 1c), which establishes the zero of time, excites the iodine molecules to the bound B state. This event prepares an ensemble of molecules coherently in one (or more) vibrational states, fixing the starting point of the oscillatory motion. Subsequent interrogation is carried out at successive (clocked) time delays by the second femtosecond laser pulse (λ_2^* in Fig. 1c).

As the molecule vibrates, the internuclear separation of the iodine atoms changes. At a certain point along the vibrational coordinate R , the probe light is absorbed and the molecule is excited to a specific vibrational level in an upper electronic state, which is determined by the Franck-Condon principle. This upper electronic state is fluorescent, and we monitor this laser-induced fluorescence (LIF) as a function of the time delay between the pump and probe pulses. Thus, as the atoms depart from and return to their initial positions over one vibrational period, the LIF signal varies accordingly, and we obtain a record of how R varies with time. If the vibrational motion is harmonic, only one frequency of oscillation will be observed (see Fig. 1a). For anharmonic vibration, on the other hand, the frequency of oscillation depends on the energy. This would result in a simultaneous excitation of a superposition of these frequencies (that is, a wave packet), as shown in Fig. 2.

To observe molecular rotations in real-time, it is necessary to establish more than just the zero of time. The pump pulse must excite the ensemble of molecules so that the subsequent rotational evolution can be followed as illustrated in Fig. 1b. To do this we use a polarized laser pulse^{9–10} to prepare the iodine molecules coherently in different rotational (J) levels. Only those molecules that have their transition moment μ (which is parallel to the internuclear axis) parallel or nearly parallel to the polarization plane of the electric field, E , are excited, because the transition amplitude is determined by $\mu \cdot E$. This anisotropic excitation of the ensemble establishes the initial alignment. The polarized femtosecond probe pulse can be fixed either parallel or perpendicular to these initially aligned iodine molecules. As the probing light is sensitive to the same transition moment (that is, both the pump and probe processes are parallel transitions), the LIF signal at early times (when very little rotation has taken place) will be large for the parallel case and small for the perpendicular one. This is illustrated in Fig. 1b and can be seen very clearly in the experimental results of Fig. 3a. As the rotation of the ensemble evolves, higher J levels rotate more quickly than lower J levels and the coherence decays. The broader the distribution of rotational levels accessed by the pump pulse, the faster this coherence disappears through dephasing of the alignment^{11,12}.

The iodine molecules are isolated and freely rotating, so a rephasing of the alignment (a rotational recurrence) should be seen at later times. The recurrence time is determined solely (if we ignore centrifugal distortion) by the rotational constant, B ,

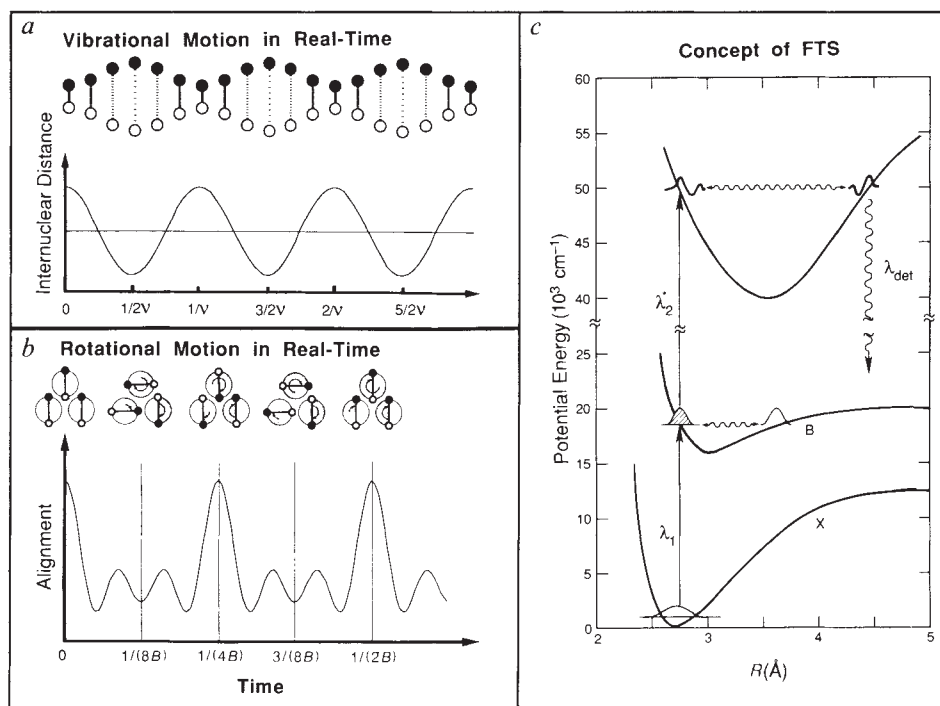


FIG. 1 a, Single vibrational (harmonic) motion, with the expected change, in real-time, of the internuclear distance, R . The peaks and valleys correspond to a fully compressed and fully extended bond, respectively. b, Molecular rotation for an ensemble of three idealized rotors, showing the initial dephasing and the half and full recurrence of the alignment at later times (see text). It should be noted that a quantum-mechanical picture^{11,12} of rotations predicts that the half recurrence at $1/(4B)$ is 180° out of phase (negative signal). The shaded region of the circles denotes the amount of rotation that the molecules have executed for each time step. The atoms are shown as black or white for clarity. c, Energy-level diagram for FTS. The first femtosecond laser pulse (λ_1) excites the iodine molecules to the $B \ ^3\Pi_{0+}$ state, with accompanying vibrational and rotational excitation. The probing is carried out by a second femtosecond laser pulse (λ_2^*), which takes them to the upper fluorescent state. The signal detected (λ_{det}) is fluorescence from the upper electronic state. Absorption or ionization at λ_2^* can also be used.

of the particular vibrational level accessed, being given by $1/(2B)$. Therefore, a rotational recurrence will be seen for each vibrational level accessed; in Fig. 1b the result is shown when only a single vibrational level is excited, whereas experimentally (Fig. 3) several are accessed. The spacing between the rotational recurrences is determined by the difference in the values of B for each vibrational level involved. Thus, rotational motions are manifested in two ways: an early-time dephasing owing to the initial loss of alignment and a longer-time recurrence owing to rephasing of this alignment.

In Fig. 2a we show vibrational transients obtained by exciting iodine molecules at room temperature into the B state using a

620-nm pump beam and probing at 310 nm. The transients are free from rotational effects, which is achieved by orientating the probe polarization at the magic angle (54.7°) with respect to the pump^{11,12}. The most striking feature of this figure is the fast oscillations with a period of 300 fs; this corresponds to the vibrational period. Their amplitude is modulated with a much longer period of 10 ps. This behaviour is due to the anharmonicity of the vibration, manifested by the interferences of the different vibrational frequencies in the wave packet.

Direct comparisons can be made with these real-time experiments, because the high-resolution spectroscopy of iodine has been studied extensively¹³⁻¹⁶. Femtosecond excitation at 620 nm

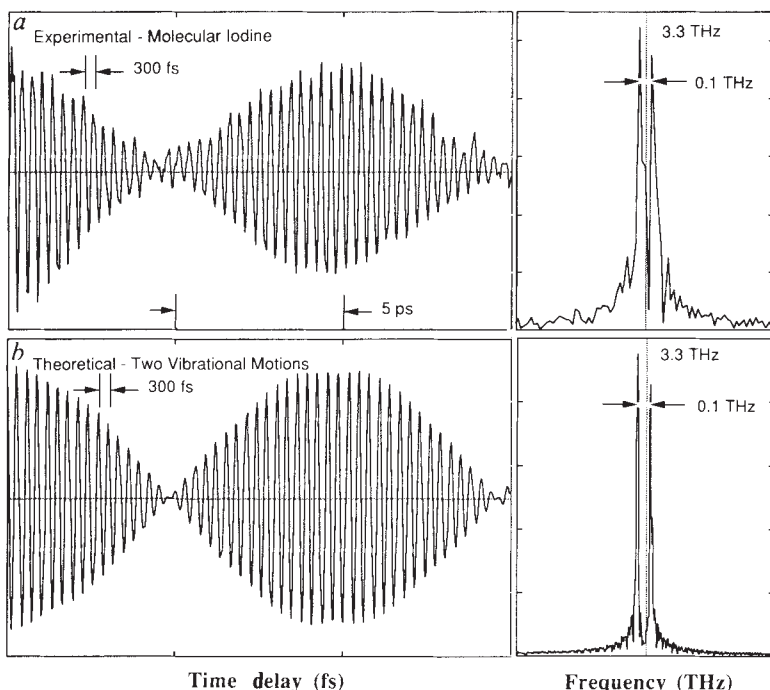


FIG. 2 a, FTS transients showing real-time vibrations of molecular iodine (left). The vertical scale represents the LIF amplitude, which is a probe of the internuclear distance. The corresponding Fourier transform is shown on the right. b, Calculated results for excitation of a wave packet comprising the two principal vibrational frequencies in a, and the corresponding Fourier transform.

should access vibrational levels 7 to 12; because of anharmonicity, the vibrational frequencies of these levels differ slightly and the average frequency should be 3.3 THz (refs 13–16), which is in excellent agreement with our results. The Fourier transform of the rotation-free transient is also shown in Fig. 2a and is consistent with the analysis above. Closer inspection of the transform reveals several peaks, corresponding to the several vibrational levels excited. The frequencies of the two largest peaks were used to simulate the transient (Fig. 2b). The complete transform has been used to obtain the potential-energy surface governing the motion of the two iodine atoms, and this analysis will be published elsewhere (M.D., R.M.B., A.H.Z., M. Gruebele and G. Roberts, manuscript in preparation). In Fig. 3 we show the results obtained for rotational motion. These were obtained under conditions identical to those of Fig. 2 except that the relative polarizations of the pump and probe pulses were fixed to be either parallel or perpendicular, so as to monitor the alignment. The initial loss of alignment is seen clearly in the early-time behaviour of the parallel and perpendicular transients. The amplitudes, initially different, approach a constant value within the first 3 ps. At much longer times (~ 600 ps), rotational recurrences are seen for each of the six vibrational levels accessed. The vibrational and rotational motions are clearly separable in time, because their periods (~ 300 fs and ~ 600 ps respectively) differ by three orders of magnitude. The fact that each vibration gives rise to a rotational recurrence (as in Fig. 3) means that rotational constants can be obtained for each vibrational level. A calculation of the expected results, taking into account the known rotational constants^{13–16} and the centrifugal distortion^{13–16} (that is, the changes in the equilibrium bond distance resulting from rotation), for the vibrational levels 7 to 12, is shown in Fig. 3b. The agreement with experiment is excellent. Finally, theory^{11,12} predicts that parallel and perpen-

dicular recurrences should be 180° out of phase and that a half recurrence at $1/(4B)$ should occur. The former effect is seen in Fig. 3a and the latter has been observed (not shown in Fig. 3); both are in accord with theory.

The FTS experiments reported here provide direct observations of the vibrational and rotational motions in isolated molecular systems. The method allows the quantum-mechanical wave-packet dynamics to be studied in real-time and related to the molecular potential-energy surface. In the early stages of FTS, the focus was on reactive processes (bond breaking or forming), but this time-resolved spectroscopy can now be applied to bound molecular systems. $\square$

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Polyethylene oxide does not necessarily aggregate in water

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POLYETHYLENE oxide (PEO) is one of the most extensively studied of all water-soluble synthetic polymers, both for its wide range of applications^{1–4} and from the fundamental standpoint of understanding the behaviour of polymer solutions. The aggregation behaviour of PEO in water and its consequences have been a matter of concern in many studies^{5–11} of this system. It is not clear, however, whether PEO aggregation is an inherent property of these aqueous solutions, although this knowledge can have important and widespread consequences for both industry and polymer science. Here we use dynamic light scattering from high-molecular-weight PEO in carefully purified water and in methanol to show unambiguously that both are good solvents in the sense that PEO behaves like a typical random coil in both, with no evidence of aggregation.

The hypothesis that aggregation is an inherent property of PEO/water systems has been supported both by electron microscopy investigations⁹ and by the observation of time-dependent PEO aggregates in aqueous solutions of the polymer on the basis of light-scattering studies¹⁰. This is in contrast to the use of methanol as the solvent—methanol is known to be a dispersing solvent for PEO^{11,12}. Furthermore, discrepancies in solvent quality, as recorded by differences in the value of the exponent a in the relation between molecular weight (M) and intrinsic viscosity (η), $[\eta] \propto M^a$, have been ascribed to aggregation of the polymer in water¹³. To clarify these issues and to test the 'aggregation hypothesis', we have carried out parallel dynamic light-scattering measurements of linear polyethylene oxide both in methanol and in water.

Dynamic light scattering is a non-invasive and accurate technique for the measurement of the diffusion coefficient D of

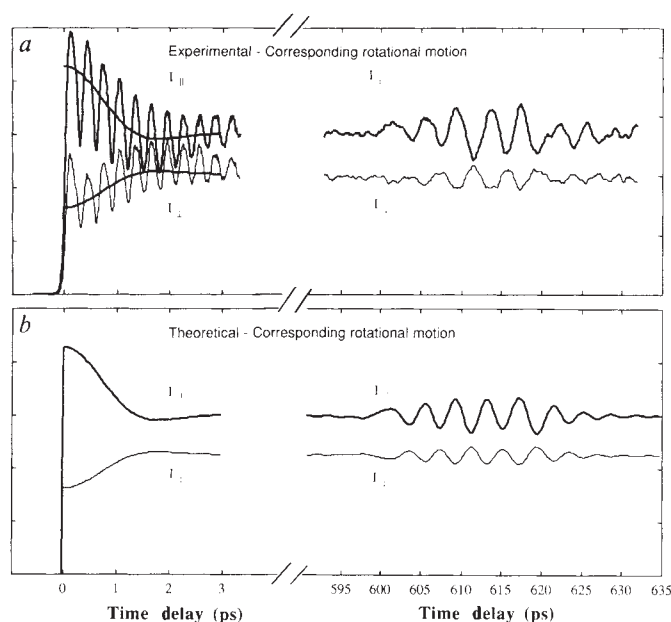


FIG. 3 Polarized FTS of molecular iodine. *a*, Transients showing the initial dephasing owing to molecular rotation (left) and the appearance of full recurrences at a later time (right). Parallel and perpendicular probe orientations are shown (see text), and are 180° out of phase, as expected. The rapid oscillations are due to vibrational excitation; six levels are accessed (the solid line through these on the left is a guide to the eye). Half recurrences were observed experimentally, but are not shown here. Note that the time scale is discontinuous. *b*, Calculated results for rotational dephasing (left) and rephasing (right), assuming excitation of vibrational levels 7 to 12 and taking into account vibrational and rotational coupling and centrifugal distortion.

TABLE 1 Polydispersity factors for PEO solutions

$M_w: 1.6 \times 10^5(1, \blacklozenge)$ $C(\text{mg ml}^{-1})$ $\mu_2/\bar{\Gamma}^2$		$M_w: 2.52 \times 10^5(2, \bullet)$ $C(\text{mg ml}^{-1})$ $\mu_2/\bar{\Gamma}^2$		$M_w: 5.94 \times 10^5(3, \blacktriangle)$ $C(\text{mg ml}^{-1})$ $\mu_2/\bar{\Gamma}^2$		$M_w: 9.96 \times 10^5(4, \blacktriangledown)$ $C(\text{mg ml}^{-1})$ $\mu_2/\bar{\Gamma}^2$	
Methanol							
2.22	0.03±0.02	1.66	0.04±0.02	0.66	0.07±0.02	2.06	0.07±0.03
1.66	0.06±0.02	1.24	0.05±0.02	0.49	0.07±0.02	1.54	0.04±0.02
1.11	0.05±0.01	0.83	0.05±0.03	0.33	0.08±0.03	1.03	0.06±0.02
—	—	0.42	0.07±0.03	—	—	0.51	0.12±0.02
—	—	—	—	—	—	0.33	0.07±0.01
Water							
0.61	0.03±0.02	2.32	0.10±0.02	1.41	0.09±0.01	2.03	0.14±0.05
1.16	0.06±0.02	1.74	0.07±0.02	1.06	0.10±0.02	1.63	0.08±0.01
1.74	0.05±0.01	1.16	0.04±0.02	0.70	0.05±0.02	1.22	0.10±0.02
2.32	0.06±0.01	0.58	0.05±0.02	0.35	0.06±0.02	0.81	0.07±0.04

Symbols correspond to those in Fig. 1.

particles in dilute solution¹⁴. By measuring the diffusion coefficient at several concentrations in the dilute regime and extrapolating to zero concentration, one can determine the diffusion coefficient for the isolated coil, D_0 , and hence the universal scaling exponent ν in the relation $D_0 = AM^\nu$, where A is a constant characteristic of the system. For small scattering wave vectors q , the diffusion coefficient corresponds to the centre-of-mass of individual brownian particles in random thermal motion¹⁴. D is obtained from the decay constant of the autocorrelation function of quasielastically scattered light at any given small q , using the relation $\Gamma = Dq^2$. The experimentally measured quantity—the second-order autocorrelation function $G^2(\tau)$ of the intensity of scattered light—yields the first-order autocorrelation function $|G^1(\tau)|$ through the well-known Siegert relation¹⁴. For monodisperse particles, $|G^1(\tau)|$ is an exponentially relaxing function. However, if there is a distribution of particle sizes¹⁵,

$$|G^1(\tau)| = e^{-\bar{\Gamma}\tau} [1 + \mu_2/2! \tau^2 + \mu_3/3! \tau^3 + \dots]$$

where $\bar{\Gamma} = \bar{D}q^2$, $\bar{D}$ is an average diffusion coefficient and the μ_n are related to the moments of the polymer molecular-weight distribution. The dimensionless polydispersity factor $\mu_2/\bar{\Gamma}^2$ is a measure of the width of the particle size distribution. For samples having narrow, symmetric molecular-weight distributions of the type used here, the polydispersity factor has been shown^{16,17} to be related to the conventional polydispersity, M_w/M_N (where M_w and M_N represent the weight- and number-average molecular weight respectively), by $\mu_2/\bar{\Gamma}^2 = \nu^2(M_w/M_N - 1)$. This number is of central importance to our study because it tells us about the aggregation of particles: aggregation would give rise to relatively large polydispersity

factors. Moreover, by comparing polydispersity factors obtained from PEO in both water and methanol (which is known to disperse PEO), an assessment of the role of water in PEO aggregation was possible.

The solutions were prepared from four TSK (Toyo Soda, Tokyo, Japan) standard polyethylene oxide samples with molecular weights ranging from 160,000 to 996,000. PEO solutions were filtered directly into sample cells through 0.2- μm (nominal pore size) Millipore fluoropore filters or Schleicher and Schuell Nylon 66 filters. Ultrapure water with a nominal resistivity between 12 and 18 $\text{M}\Omega\text{ cm}$ was used for preparation of the aqueous solutions¹⁸. The pH of the water was ~ 5.5 . For the aqueous stock solutions, a drop of chloroform was added to prevent the growth of bacteria; therefore we compared results obtained with and without the addition of a drop of chloroform, finding that within experimental uncertainties, the presence of chloroform did not affect our results. The dynamic light-scattering measurements were carried out using the laser-light-scattering photometer/goniometer system described in detail in ref. 19. For the sample with highest molecular weight, measurements were made at four different scattering angles to ensure that internal modes of motion did not contribute to the measured correlation function¹⁴.

Table 1 presents average values of the polydispersity factor obtained for all sets of measurements. Typical values lie around 0.05 and there is no evidence to suggest that these values are higher when water rather than methanol is used as the solvent. Corresponding polydispersity factors obtained from the dynamic light-scattering measurements presented in ref. 10 are much higher, even in methanol. Those authors stated, however, that their solutions were neither filtered nor centrifuged.

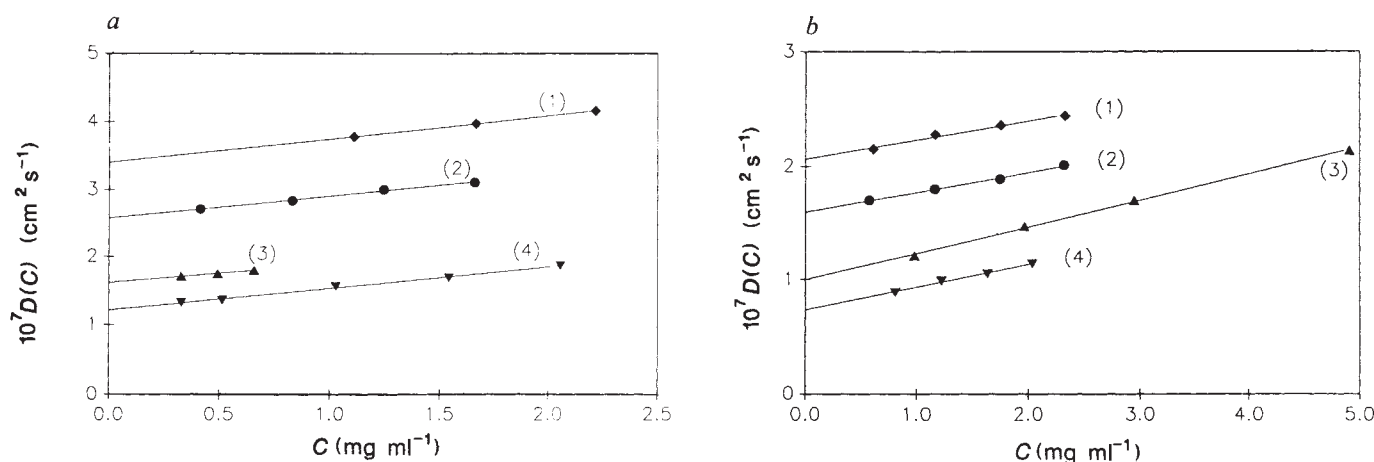


FIG. 1 Plots of the concentration dependence of the PEO diffusion coefficients, for a, PEO/methanol, and b, PEO/water. (See Table 1 for symbol designations.)

Figure 1 shows the dependence of PEO diffusion coefficients on concentration in both solvents. The positive slopes indicate the good solvent nature of both systems. This conclusion is supported by the value of the scaling exponent ν in the relation between D_0 and the weight-averaged molecular weight: the value is $\nu = 0.56$ for both solvents, consistent with that for a swollen coil in a good solvent²⁰. Intrinsic viscosity measurements^{21,22} support the fact that water at 30 °C and at 25 °C is indeed a good solvent ($a = 0.78$) for high-molecular-weight PEO. Measurements of the intrinsic viscosity of aqueous solutions of PEO as a function of molecular weight (D. Woodley, C. Dam, K.D. & J.C.S., manuscript in preparation) also confirm that water is a good solvent at 30 °C ($a = 0.78$). To compare our results with those made at 25 °C, the values of D_0 for the $M_W = 594,000$ sample were measured at 25 °C and 30 °C. After correction for solvent viscosity and temperature differences, we obtained comparable values of D_0 within experimental uncertainty, illustrating that water is an equally good solvent at both temperatures.

Figure 2 shows the dependence of D_0 and of the hydrodynamic radius, $R_H \sim 1/D_0$, on molecular weight for the two solvents. It is immediately obvious that the PEO random coil occupies a larger volume in water than in methanol, but the essentially identical slopes indicate that aggregation is not responsible for this. There seem to be two possibilities for this intriguing size difference. Raman spectroscopy and NMR measurements of PEO in water suggest that water molecules attach to the oxygen atom in the PEO chain^{23,24}. This type of association is unlikely in methanol^{23,24}. Also, based on the partial

specific volume of polyethylene oxide in water, the polymer has a greater compacting effect on water than on any other solvent². If water is packed within the PEO coil, this could explain the larger hydrodynamic radius. The other possibility is based on evidence of local helicity in PEO when water is used as the solvent^{25,26}. If this helicity does indeed exist, the chain is likely to be less flexible in water, which can lead to a slight increase in the overall dimensions of the coil.

The fact that PEO can exist without aggregating in water has important practical as well as fundamental consequences. For example, it is believed that an entangled or adsorbed layer of PEO is responsible for its drag-reducing properties in the flow of water through ducts and channels⁵⁻⁸. If aggregation is desirable for such drag-reduction applications, our results now provide the 'unaggregated state' as their reference point: from the dispersed state, aggregation could be induced in a controlled way, if its dependence on relevant parameters such as pH, ionic strength and so on are carefully assessed and controlled. For example, we find that even an extremely low concentration of organic impurities ('dust') in PEO solutions results in aggregation. It seems that the presence of very minute amounts of such impurities can readily shift the thermodynamic coordinates of such a solution so as to approach polymer association and phase separation, for example. A detailed understanding of the mechanisms of aggregation will emerge, however, only after considerable additional work. At present, there exist no more than plausible explanations (such as hydrogen bonding, the hydrophobic effect, the presence of organic and inorganic impurities) for this complex behaviour.

Earlier studies of local phenomena in PEO solutions using NMR^{23,24}, infrared²³ and Raman²⁶ spectroscopies have revealed that PEO in water retains local helical structure. Thus, in certain important respects, PEO can serve as a synthetic analogue of DNA. Consequently, single-chain behaviour important both in DNA and PEO—such as the effect of backbone hydration on chains stiffness and on coil internal dynamic behaviour, and salt- or pH-induced coil shrinkage and 'collapse'—may be studied using PEO in water.

In conclusion, we have shown that there is no reason to believe that aggregation is an inherent property of polyethylene oxide/water systems. Polymer theory pertaining to binary solutions is therefore applicable to studies of PEO behaviour in water, and unaggregated PEO solutions provide a reference state for the further investigation of PEO aggregation. □

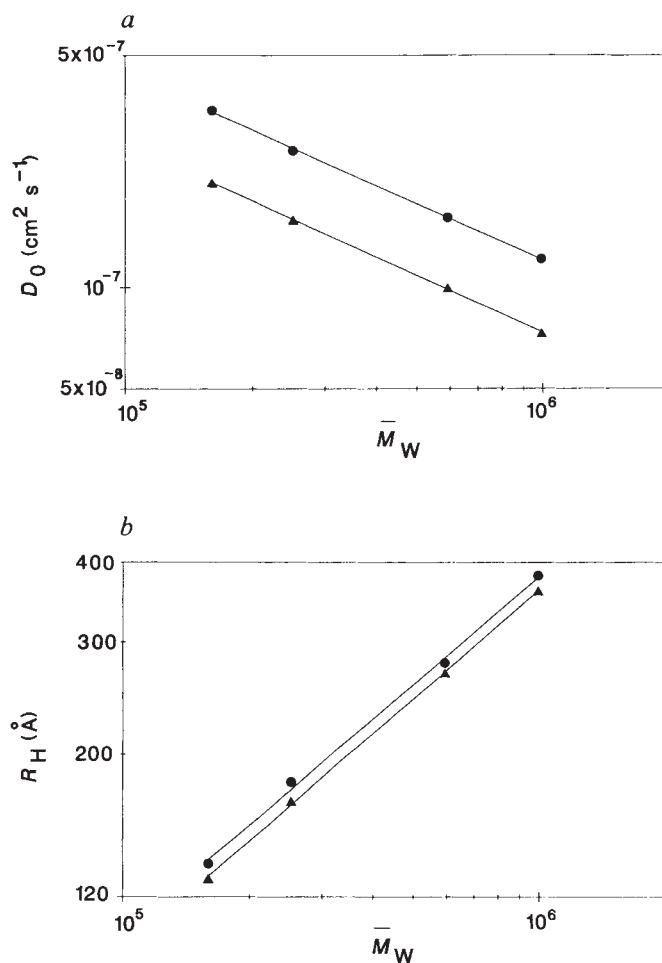


FIG. 2 Dependence of a , the PEO infinite-dilution diffusion coefficient, D_0 , and b , the PEO hydrodynamic radius, R_H , as functions of the weight-average molecular weight, M_W .

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Diamond inclusions in garnets from metamorphic rocks: a new environment for diamond formation

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DIAMONDS commonly occur in kimberlites, lamproites and alluvial sediments derived from these rocks. More recently, diamonds (or their graphite pseudomorphs) have been discovered in ultramafic massifs¹ and picrites². Here we report the occurrence of diamonds *in situ* in crustal rocks: highly retrograded high-pressure metamorphic garnet–pyroxene and pyroxene–carbonate–garnet rocks, biotite gneisses and schists from the Kokchetav massif, northern Kazakhstan, USSR. The diamonds are cubo-octahedral, averaging 12 μm in size, and occur in zircons, and with euhedral graphite as inclusions in unzoned garnets. We believe that the zircon and garnet matrices protected these diamonds from retrogressive transformation to graphite. Mica, rutile, titanite, clinopyroxene, kyanite and zircon also occur as inclusions in garnet, often intergrown with the diamonds. Equilibration relations of inclusions and host garnets indicate that both diamonds and graphite crystallized from a fluid phase under static conditions at pressures of ≥ 40 kbar and temperatures > 900 – $1,000^\circ\text{C}$.

The Kokchetav Massif is a large ($\sim 60,000$ km²) fault-bounded body of Proterozoic metamorphic rocks surrounded by Caledonian rocks of the Ural–Mongolian fold belt. It is a part of the Central Asian Belt, the major collision zone of Asia³. Geological relationships indicate that the massif was emplaced near the end of the Precambrian⁴. The massif core, where diamondiferous rocks occur, consists of a variety of crystalline schists and gneisses, eclogites, pyroxene granulites, amphibolites, quartzites, marbles and associated calc-

magnesite–silicate rocks of amphibolite and granulite facies. Granitic rocks constitute over $\sim 65\%$ of the present exposure. The mineralogy of the eclogites has recently been reviewed⁵, and suggests equilibration conditions of 900°C , ≥ 18 kbar, for the eclogites associated with the diamond-bearing rocks, and 600 – 700°C , 12 – 14 kbar, for eclogites in the eastern portion of the massif.

A garnet–clinopyroxene Sm–Nd age of 533 ± 20 Myr dates the end of high-pressure metamorphism. The diamondiferous rocks seem to have differentiated > 2 Gyr ago based on interpretation of a whole-rock Pb–Pb isochron⁶.

The diamonds occur mainly as inclusions in garnets in garnet–pyroxene, pyroxene–carbonate–garnet rocks, garnet–biotite gneisses and schists. These rocks occur as small lenticular, banded or vein-like bodies within plagioclase gneisses of the Zerendinsk rock series.

Garnet–pyroxene rocks are coarse-grained with euhedral to subhedral garnet (20–40 modal %) and clinopyroxene (50–80%), with interstitial calcite. Accessory minerals include rutile and titanite, secondary chlorite, quartz and K-feldspar. The pyroxene–carbonate–garnet rocks are characterized by large porphyroblasts of clinopyroxene, which are set in a slightly cataclastic matrix of mica and calcite. Garnet grains (up to 1 mm in size, 1–3 modal %) occur randomly throughout the rock.

Schists and gneisses are medium-grained rocks with widely varying modes of plagioclase, quartz, garnet, K-feldspar, biotite and chlorite. Graphite, zircon, apatite and sulphides are accessory. The diamonds are distributed heterogeneously within a given rock and seem to be concentrated in zones or layers, which are otherwise undistinguished in mineralogy. Garnet is relict and is usually replaced by biotite, K-feldspar and chlorite; plagioclase is locally saussuritized. In some samples several grains of clinopyroxene are present, which are partly altered to amphibole and chlorite. Kyanite is also sometimes observed.

Identification of diamond inclusions was made by optical microscopy and Raman spectroscopy on 0.3-mm-thick doubly polished plates of garnet-bearing rocks, as well as by X-ray diffraction and scanning electron microscopy of inclusions extracted from the host garnet. Mineral analyses were obtained

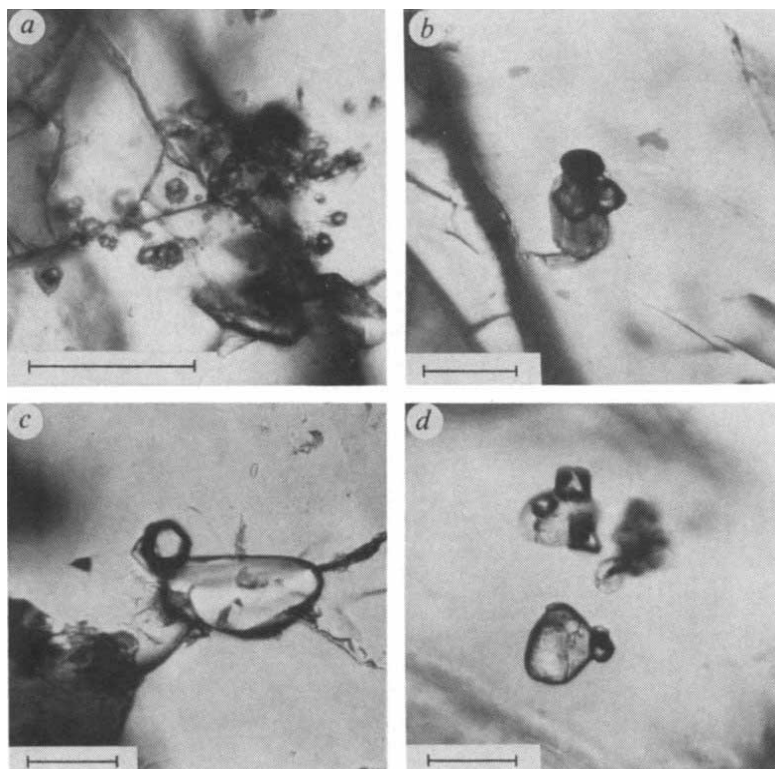


FIG. 1 Diamond inclusions in garnets from metamorphic rocks. *a*, Abundant diamonds intergrown with mica flakes. *b*, A single mica flake with two diamonds and a graphite crystal (dark). *c* and *d*, Diamond intergrowths with rutile. Scale bar is $40\ \mu\text{m}$ for all photographs.

TABLE 1 Representative mineral analyses from diamond-bearing rocks

	Garnet-pyroxene rock (81/5)		Pyroxene-carbonate rock (81/21)		Biotite-garnet plagiogneiss (83/3)			
	gt	cpx	gt	cpx	gt _c	gt _r	bi	musc
SiO ₂	38.8	51.7	40.5	55.5	39.5	39.4	35.8	46.9
TiO ₂	0.23	0.02	0.25		0.13	0.13	2.27	1.21
Al ₂ O ₃	21.5	2.91	22.1	1.47	21.9	22.3	17.6	30.5
FeO	14.9	5.05	5.58	1.58	18.8	17.8	10.4	1.11
MnO	0.86	0.22	0.35	0.01	0.81	0.82		
MgO	7.43	14.3	10.9	17.8	7.45	7.98	14.9	2.21
CaO	15.6	22.5	19.1	24.1	11.1	12.2	0.04	
Na ₂ O		1.03		0.19			0.24	0.27
K ₂ O							9.42	9.94
Total	99.29	97.73	98.76	100.65	99.69	100.33	90.67	92.14
Oxygen	12	6	12	6	12	12	22	22
Si	2.967	1.946	3.000	1.988	3.013	2.974	5.495	6.434
Ti	0.013	0.001	1.928		0.008	0.007	0.262	0.125
Al	1.938	0.129	0.014	0.062	1.969	1.984	3.184	4.932
Fe ²⁺	0.953	0.159	0.288	0.047	1.199	1.237	1.335	0.127
Mn	0.056	0.007	0.022	0.001	0.052	0.052		
Mg	0.847	0.802	1.199	0.951	0.847	0.898	3.409	0.452
Ca	1.278	0.907	1.515	0.925	0.907	0.987	0.007	
Na		0.075		0.013			0.071	0.072
K							1.845	1.740
Σ	8.051	4.026	4.012	3.987	7.995	8.026	15.608	13.881
Fe/(Fe + Mg)	53.0	16.5	22.3	4.7	58.6	55.6	28.1	22.0
Ca-comp	41.5	48.6	50.5	49.5	30.4	32.5		

	Garnet-amphibole-biotite rock (4/19)				Biotite-garnet gneiss (53)					
	gt _c	gt _r	amph	bi	cpx	plag	k-fsp	bi	gt _c	gt _r
SiO ₂	39.7	39.4	43.4	38.0	53.5	63.8	65.9	38.9	39.4	39.4
TiO ₂	0.16	0.21	0.43	1.76	0.13			1.53	0.11	0.04
Al ₂ O ₃	21.7	21.6	13.8	15.5	4.28	21.8	18.5	15.0	21.4	21.4
FeO	20.9	21.9	11.9	14.6	7.22			15.0	21.3	20.1
MnO	1.49	1.87	0.23	0.41	0.12			0.27	1.18	1.43
MgO	7.51	6.93	12.4	16.2	12.3			15.7	4.5	3.6
CaO	9.16	9.33	10.9	0.03	21.7	4.16			12.7	14.4
Na ₂ O			1.48	0.7	1.48	7.75	1.10	0.14		
K ₂ O			1.25	9.41		0.83	14.4	9.16		
Total	100.62	101.24	95.69	95.98	100.73	98.34	99.90	95.70	100.59	100.37
Oxygen	12	12	23	22	6	8	8	22	12	12
Si	3.020	3.001	6.466	5.593	1.958	2.857	3.017	5.752	3.032	3.040
Ti	0.009	0.012	0.048	0.195	0.004			0.170	0.006	0.002
Al	1.946	1.939	2.423	2.689	0.185	1.151	0.998	2.614	1.941	1.946
Fe ²⁺	1.330	1.395	1.483	1.797	0.221			1.855	1.371	1.297
Mn	0.096	0.121	0.029	0.051	0.004			0.034	0.077	0.094
Mg	0.851	0.787	2.754	3.554	0.671			3.460	0.516	0.414
Ca	0.747	0.762	1.740	0.005	0.851	0.200			1.047	1.191
Na			0.428	0.200	0.105	0.673	0.098	0.040		
K			0.238	1.767		0.047	0.841	1.728		
Σ	7.998	8.017	15.607	15.851	3.998	4.928	4.953	15.654	7.991	7.984
Fe/(Fe + Mg)	60.9	64.0	35.0	33.6	24.8			34.9	72.6	75.8
Ca-comp	25.1	25.2			48.8				35.9	41.3

gt, Garnet; gt_r, garnet rim; gt_c, garnet core; cpx, clinopyroxene; bi, biotite; musc, muscovite; amph, amphibole; plag, plagioclase; K-fsp, K-feldspar.

using standard electron-microprobe techniques with a CAMEBAX-MICRO microprobe.

In all diamond-bearing rock types the diamonds occur as inclusions in zircons and garnets, either as single grains or groups of grains. Within the garnets, diamonds are often intergrown with euhedral biotite, zircon or rutile (Fig. 1). We did not observe diamonds elsewhere. Almost 90% of the diamonds occur in association with mica. In the biotite gneisses, titanite, phengite, K-feldspar, quartz and kyanite also occur as garnet inclusions.

Graphite is present in all the diamond-bearing rocks described above and occurs as: (1) inclusions in garnet (~50 µm), locally intergrown with mica, or on the surface of diamond crystals, and (2) secondary crystals (≤300 µm) in intergranular areas, or in fractures that cut the major minerals.

Garnet compositions in the gneisses and pyroxene rocks are similar to those in the eclogites⁵ (Table 1). All contain a large

grossular component with low Fe/(Fe + Mg), particularly those from the garnet-pyroxene and the pyroxene-carbonate-garnet rocks (see Table 1). The composition of these garnets approaches that of the grosspyrites from kimberlitic pipes⁷. None of the garnets are zoned.

Pyroxenes fall into the diopside-hedenbergite range and have very low Al₂O₃ contents (Table 1). Pyroxenes in the pyroxene-carbonate rocks are almost pure diopside, whereas those from the gneisses and schists contain moderate amounts of jadeite, usually no more than 10%.

A pyroxene inclusion in garnet from the pyroxene-carbonate-garnet rock contains an extremely high K₂O content (Table 2), in contrast to pyroxene in the host rocks (Table 1). This high K₂O content suggests crystallization at high pressures⁷.

Biotite intergrown with diamond in garnet from the garnet-biotite gneiss has a lower Fe/(Fe + Mg) compared with biotite

in the host rock (Tables 1, 2; sample 83/3). Biotite inclusions in one sample (258, Table 2) contain ~5 wt% F and Cl. Rutile inclusions (Table 2) contain 0.17 wt% Al_2O_3 , which is in the range of rutiles in high temperature eclogites from kimberlites⁷. Titanite inclusions in garnet (Table 2) have high contents of Al_2O_3 and F.

The average diamond size is $12.5 \pm 6.0 \mu\text{m}$ ($n = 501$); 80% of all diamonds are $< 20 \mu\text{m}$. We determined the morphologies of the diamonds (Fig. 2) both *in situ* and after extraction. The majority of the diamonds are cubo-octahedra, with varying development of cube and octahedral faces (Fig. 2). Features such as laminar growth on octahedral faces (Fig. 2a), overstepping growth of cube surfaces on octahedral faces (Fig. 2b, c) and fibrous growth (Fig. 2d) are visible. In some instances the cube surfaces are frosted by many tetragonal etch pits (Fig. 2e). Polycrystalline aggregates of cubo-octahedral crystals of varied sizes also occur (Fig. 2f).

Although occurring in metasedimentary rocks, the micro-diamonds are not likely to be detrital because: (1) diamonds have not been found outside of zircon or garnet, both of which are high-grade metamorphic minerals. Detrital diamonds are unlikely to have survived prograde metamorphism; (2) diamonds are intimately intergrown with biotite flakes (Fig.

1a, b), which are clearly not detrital; (3) diamond aggregates (Fig. 2f) are extremely delicate and could not have survived sedimentary transport; (4) other high-pressure inclusions within garnet (particularly the high- K_2O clinopyroxene) are also unlikely to have survived sedimentary transport and thus reflect a previous ultra-high-pressure metamorphism.

It is difficult to fix the precise pressure and temperature conditions under which the diamond-garnet assemblages formed because of the subsequent retrograde metamorphism of the rocks. Nevertheless, there are some independent indications of high pressure. In the associated titanite inclusions, for example, Al occupies 33% of the Ti sites (Table 2; compare with ref. 8). These titanite compositions are similar to those found in high-pressure eclogites from Norway (which crystallized⁹ at ~25 kbar), Italy and Austria¹⁰.

Garnet of low Fe/Fe+Mg and high grossular content, comparable to that containing diamonds in this study, has been shown to be stable at pressures exceeding 25 kbar. Furthermore, the presence of K-feldspar lamellae in clinopyroxenes and high-K pyroxene inclusions in garnet (Table 2) is consistent with their formation at high pressures⁷.

The above evidence indicates that the diamonds in these metamorphic rocks crystallized *in situ* in the diamond stability

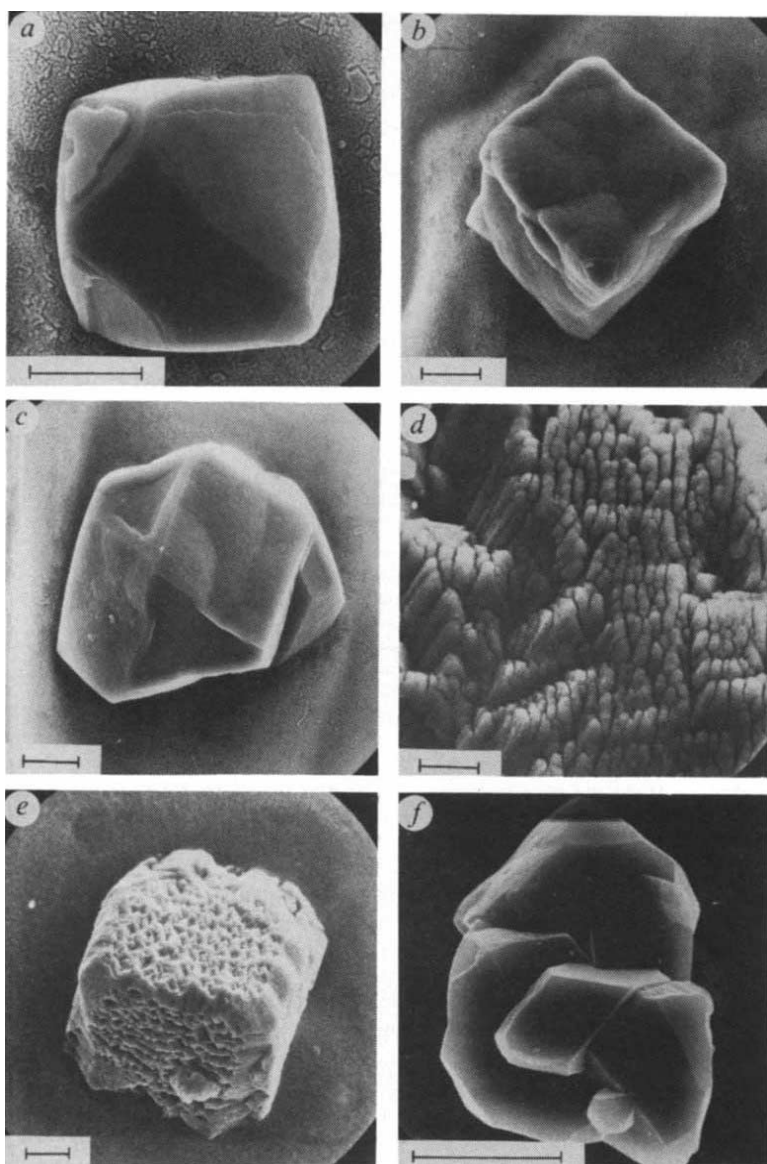


FIG. 2 Morphological types of diamond inclusions (see text for explanation). Scale bar is $10 \mu\text{m}$ for all photographs except d, in which the scale bar is $1 \mu\text{m}$.

TABLE 2 Composition of mineral inclusions in garnet

Sample	83/3		81/21		4/19	258
	rutile	cpx	bi	bi	bi	titanite
SiO ₂	0.05	54.9	40.6	39.9	39.2	32.0
TiO ₂	97.2	0.03	0.97	1.81	1.81	22.1
Al ₂ O ₃	0.17	2.62	15.6	16.5	14.0	11.7
FeO	0.62	1.21	5.00	9.18	12.5	0.88
MnO		0.06	0.04	0.21	0.12	0.05
MgO		16.3	22.0	18.8	17.0	0.05
CaO	0.10	23.2		0.07	0.10	28.5
Na ₂ O		0.42	0.01	ND	0.16	ND
K ₂ O		1.14	10.3	8.01	9.17	
F			0.76	ND	4.46	3.99
Cl					0.44	
Total	98.14	99.88	95.28	94.48	98.96	99.27
Oxygen		6	22	22	22	5
Si		1.986	5.807	5.746	5.832	1.072
Ti		0.001	0.104	0.196	0.202	0.556
Al		0.112	2.630	2.801	2.455	0.462
Fe		0.037	0.598	1.106	1.555	0.025
Mn		0.002	0.005	0.026	0.015	0.001
Mg		0.879	4.689	4.036	3.770	0.002
Ca		0.899		0.011	0.016	1.022
Na		0.029	0.003		0.046	
K		0.053	1.879	1.472	1.740	
F			0.166		2.098	0.423
Cl					0.111	
Σ		3.998	16.058	15.393	17.841	3.564
Fe/(Fe + Mg)		4.0	11.3	21.6	29.2	

83/3, Biotite-garnet plagiogneiss; 4/19, garnet-amphibole-biotite rock; 258, biotite gneiss; 81/21, pyroxene-carbonate rock. ND, not detected.

field. Thus metamorphic pressures reached at least 40 kbar in the rocks of the Kokchetav Massif. Equilibration temperatures¹¹ for clinopyroxene inclusions and their surrounding host garnets are in the range of 900–1,000 °C.

A number of investigations have been directed towards understanding the mechanism of diamond growth through analysis of their surface morphologies^{12–15}. Surface features of the Kokchetav microdiamonds (such as cubic crystals with commonly rounded surfaces, square pyramids and pits on cuboid surfaces (Fig. 2)) are similar to those of diamonds from kimberlite and associated xenoliths^{16–18}, implying similar growth mechanisms for both. In addition, like the Kokchetav example, both octahedral and cubic diamonds have been observed to coexist with graphite in eclogite xenoliths^{17,18}.

Because the groundmass mineral assemblage has re-equilibrated at lower pressures and the mineral inclusions within garnets may or may not have partially re-equilibrated, the petrogenesis of the diamonds and associated inclusions is difficult to assess. The absence of fluid inclusions in the host garnets prevents direct determination of the fluid phases present during crystal growth. Nevertheless, fluid inclusions are present in several generations of quartz from various rocks of the Zeren-dinsk series that are associated with the diamond-bearing samples¹⁹. The earliest quartz contains the highest-density, inclusions—CH₄–H₂O–NaCl. An intermediate generation contains medium density CH₄–CO₂–H₂O–NaCl inclusions, and the latest quartz contains low-density CH₄–CO₂–H₂O inclusions. The variable compositions of fluid inclusions in rocks of close spatial relationship indicates low fluid mobility.

There are two ways in which free carbon may have formed in the Kokchetav rocks: (1) if temperature and f_{O_2} were constant, increasing pressure would cause decreasing solubility of carbon in a fluid phase, resulting in the precipitation of diamond or graphite, or (2) oxidation of a CH₄-bearing fluid phase to form water and free carbon. The heterogeneous and layer-like distribution of both diamond and graphite in these rocks favours the latter hypothesis, because metapelites may preserve variable f_{O_2} between layers.

The common association of diamond with mica in the

inclusions suggests that both minerals precipitated from a fluid phase, which can be characterized using the fluid-inclusion data. The compositions of the earliest-formed, high-density fluid inclusions indicate that the oxygen fugacity was controlled by the reaction $CH_4 + O_2 \rightarrow C + 2H_2O$. Thus the precipitation of carbon may be related to the oxidation of a CH₄ fluid. Whether this carbon occurs as graphite or diamond depends on the f_{O_2} : with increasing f_{O_2} the partial pressure of CH₄ decreases and graphite forms instead of diamond²⁰. Recent studies have demonstrated the importance of fluids in diamond formation^{21,22}.

Diamonds with similar morphology and size to those occurring in the Kokchetav massif have been reported in fine-grained Tertiary titaniferous sands from the Pridneprovie region in the western Soviet Union²³ and from northern Kazakhstan (in the same region as the Kokchetav massif)²⁴. A common feature of all these microdiamonds is their anomalously negative $\delta^{13}C$ values ($[(^{13}C/^{12}C)_{\text{sample}}/(^{13}C/^{12}C)_{\text{standard}}] - 1$), which range from –10.4 to –21.6‰ (ref. 25). Similar alluvial microdiamonds with strongly negative $\delta^{13}C$ (ranging from –15.3 to –28.1‰)²⁶ have been reported from northern Australia²⁷. Microdiamonds from the Kokchetav metamorphic rocks are depleted in ^{13}C ($\delta^{13}C \leq -8.7\%$; E. Galimov and N.S., unpublished data). The similarity of size, morphology and $\delta^{13}C$ between the alluvial diamonds and the metamorphic diamonds from the Kokchetav massif suggests that high-pressure metamorphic massifs may be the source of these alluvial diamonds.

The morphological and isotopic compositions of both the alluvial and metamorphic microdiamonds are distinct from the majority of alluvial diamonds derived from kimberlites or lamproites, which typically have more variable sizes, morphologies and a restricted range in $\delta^{13}C$ (from –4 to –8‰)^{28,29}. The anomalously negative values of the alluvial and metamorphic microdiamonds are typical of diamonds of eclogitic affinities from kimberlitic³⁰ and lamproitic^{26,31} pipes. These negative values may indicate that the carbon was derived from recycled crust.

We conclude that diamonds can form *in situ* in metamorphic massifs, and these crustal rocks may therefore be the source for some alluvial microdiamonds. This discovery, in combination with previous discoveries of coesite in the rocks of the Dora Maira complex³² and in Norwegian eclogites³³ widens the pressure-temperature field for crustal metamorphic rocks in the Earth's lithosphere^{34,35}. This in turn has important implications for tectonic models, which must explain how rocks, originally formed at the Earth's surface, were taken to depths of ~100 km, metamorphosed and returned to the Earth's surface, while retaining relicts of a high-pressure, high-temperature history. □

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Satellite tracking of Wandering albatrosses

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ALTHOUGH the study of seabirds in their land-based breeding colonies has attracted much attention, an understanding of their ecology at sea, particularly their foraging range and the location of their feeding zones, remains a major challenge¹. The foraging range of pelagic feeders nesting on a given colony or island is purely speculative^{2,3}. Since the eighteenth century⁴, the Wandering albatross (*Diomedea exulans*) has been thought to be one of the most widely ranging seabirds, and breeders are thought to travel up to 1,800 km from the nest on foraging trips^{5,6}. Here, we describe the first successful tracking of a bird using satellite telemetry. Tracks of Wandering albatrosses in the southwestern Indian Ocean showed that they covered between 3,600 and 15,000 km in a single foraging trip during an incubation shift. They flew at speeds of up to 80 km per h and over distances of up to 900 km per day. They remained active at night, particularly on moonlit nights and wind appeared to have a major influence on the foraging strategy of these albatrosses. Detailed knowledge of their movements at sea may prove critical to the conservation of the Wandering albatross and particularly of the closely related Amsterdam albatross (*D. amsterdamensis*), both of which are endangered^{7–9}.

Conventional radiotelemetry has limited applications for determining the foraging movements of widely ranging animals. The curvature of the earth and attenuation by salt water both limit signal range for pelagic animals and may necessitate either prohibitively large power sources or costly labour-intensive receiving units¹⁰. We used satellite tracking¹¹ (see Table 1 legend) to follow the movements of six male Wandering albatrosses between January and March 1989, while they were breeding on Possession Island in the Crozet archipelago in the south-western Indian Ocean. Birds (weight, 10–12 kg, wingspan, 3.0–3.2 m), were equipped with 180 g transmitters. Two individuals were initially fitted with dummy transmitters and were weighed on their return from foraging trips. They had gained 7% and 15%, respectively, of their departure weights; these values are typical of weight gains at sea¹². The birds were equipped with a transmitter and released onto the nest, where they were relieved some days later by the female. The transmitters were recovered from the birds when they returned to the nest for a new incubation stint after foraging trips lasting 2–33

days. Of six males equipped with transmitters on foraging trips, five returned to the Crozet Islands whereas one (male B) failed to breed and continued its foraging activities, at least until the transmitter's batteries were exhausted (Table 1). A total of 1,561 locations were obtained, at an average rate of 11.8 locations per day per bird (Table 1).

The birds covered between 3,664 and 15,200 km in a single foraging trip during their partners' incubation shifts (Table 1). They generally followed a looping course from Crozet, ranging in all directions as far as the Antarctic continent to the south, the subtropical waters to the north and Heard Island to the east (Fig. 1). Foraging trips were much shorter during brooding shifts; one bird (F) only covered 330 and 381 km in two successive trips, returning at a 4-day interval to the same oceanic area at the border of the Crozet continental shelf, 130 km from the colony. The Wandering albatrosses only undertook long-distance travel in the daytime (Fig. 2; Table 1), but even at night they were never recorded as being stationary for more than 1.6 h. Daytime flight distances of up to 936 km were recorded. Short-distance daytime flights of less than 100 km were mainly observed for birds at the core of a high-pressure system (Fig. 2). The birds covered much shorter distances by night (generally less than 50 km) and tended to fly further on moonlit nights than on nights with no moon (Fig. 3). Maximum flight speeds calculated between two locations were between 62.9 and 81 km per h (Table 1). The birds could sustain high average speeds over long distances, for example up to 56.1 km per h over 808 km (Table 1). A major influence on flight direction in the sector prospected by a bird proved to be wind direction, with birds almost never flying to windward (Fig. 4). Leeward winds tended to be used on outward journeys, while the birds tacked against

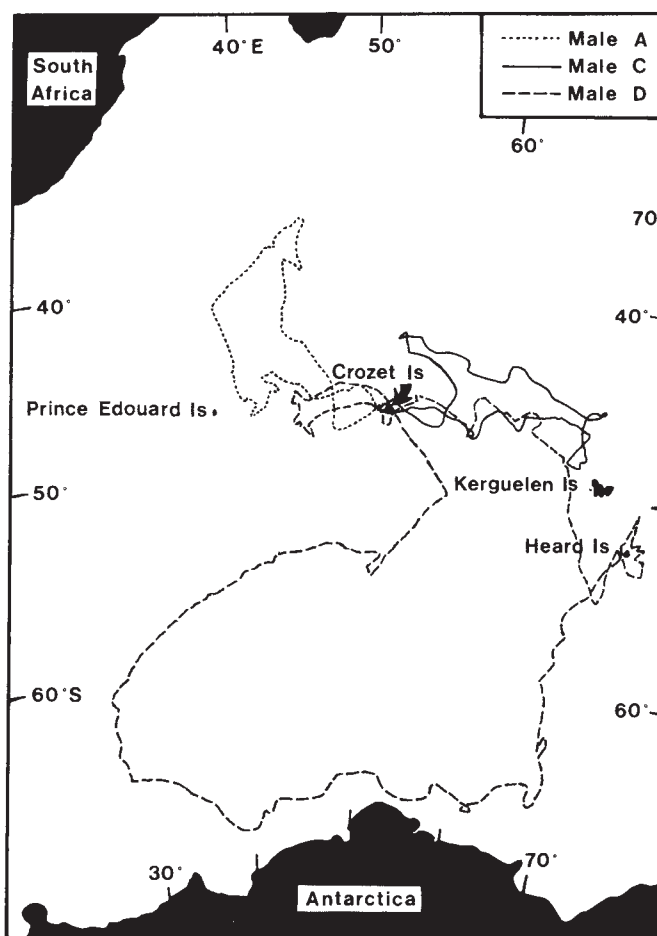


FIG. 1 Tracks of three Wandering albatrosses in the southern Indian ocean.

TABLE 1 Foraging patterns of six Wandering albatrosses

Male	Period of transmission at sea	Duration of foraging trip (days)	Number of locations	Distance covered		Speed of flight (km per h)	
				Total (km)	% in daytime	Maximum	Average/distance covered
A	19/01-12/02	24	262	5,609	87.3	68.7	55.1/446 km
B	29/01-25/02	27	314	10,427	87.1	62.7	49.2/949 km
C	01/02-25/02	24	225	5,323	88.8	73.7	54.4/463 km
D	01/02-25/02	33	385	>15,200	95.9	81.2	56.1/808 km
E	07/03-21/03	14	166	3,664	90.9	73.9	58.4/383 km
F	22/03-25/03	3	34	381	57.7	24.7	
F	30/03-02/04	3	36	330	78.5	30.1	

Five male Wandering albatrosses (A-E) were tracked during incubation and male F was tracked during two successive foraging trips during the chick brooding period. Maximum speeds of flight were calculated between two locations over periods of at least 1.6 h. The transmitter batteries of male D were exhausted after 25 days at sea, but the bird returned to relieve its mate on March 5th. We used T2028C PTT transmitters constructed by TOYOCOM Ltd, Japan, and the ARGOS System. The transmitters, which represented 1.3-2% of the bird's body weight were of tubular form with rounded ends, this shape best reduces drag in flying birds¹¹. Data from two satellites were stored at the ARGOS Processing Centre in Toulouse, France, and regularly retrieved from the Centre to be analysed. The tracks of the birds were analysed in relation to surface analysis meteorological maps of the southern Indian Ocean provided twice daily by the Weather Bureau in Melbourne, Australia.

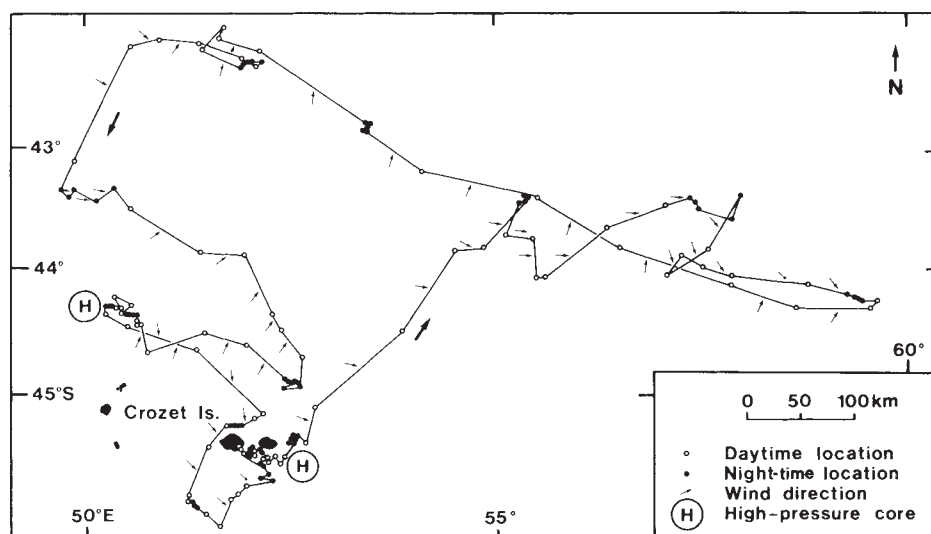
lateral winds on the return journey.

This study demonstrates the utility of satellite telemetry in determining the movements of widely ranging birds. Wandering albatrosses seemed to be unable or unwilling to navigate over long distances in complete darkness. The birds moved continuously in windy conditions. During the day they never remained in the same zone although their movements could be interrupted by short stops that probably corresponded to feedings. The birds rested at night for short periods, rarely exceeding one hour, during which they could also feed. Both the distances covered and the long-distance flight speeds were much higher than the highest estimates for this or any other species of bird^{3,13}. These flight patterns are possible because of the dynamic soaring flight of albatrosses which is based on optimal use of the wind in terms both of energy extraction from the wind and of energy saving for the bird¹³. In this respect, one of the most stimulating prospects that satellite tracking holds is to determine how foraging strategy can be optimized when weather conditions have such a large influence on the movements of great albatrosses. The birds took maximum advantage of the prevailing winds on the outward journey. The probability that they faced the wind on the return journey was high, and in this situation they either tacked in towards the island in a long zig-zag route, or they

looped around it until they encountered favourable winds (Fig. 2). High-pressure systems acted as traps that could immobilize the birds for periods of 1-7 days, at least in this study. Such systems are rare, however, and occur mainly in spring, when the birds are incubating their eggs. The high-pressure systems were probably responsible for the high level of variability (up to 40 days) in the duration of foraging trips^{5,14}. Prolonging the foraging trip in the absence of wind did not necessarily result in breeding failure because an incubating bird can fast for long periods at this stage in the breeding cycle (we have observed females fasting for up to 50 days before deserting the egg and larger males for at least 55 days). After hatching, the young chick has to be fed at frequent intervals and the foraging trips of Wandering albatrosses last for 2-4 days on average^{5,14}; the birds modify their strategy and forage close to the nesting grounds, even in the presence of favourable winds (Table 1).

Despite occasional anticyclones, the foraging zones of Wandering albatrosses lie in a region which is subject to stronger and more regular winds than in any other part of the world where low-pressure systems follow one another at short, regular intervals. Thus, throughout the year, but particularly during autumn and winter when the birds are rearing their chicks and must visit the nest most frequently, they can base their foraging

FIG. 2 Details of the foraging path taken by male E between 7 and 21 March 1989, mapped from 165 satellite locations with readings at mean interval of 1.6 h. The bird moved further between daytime locations than between night-time locations and was almost never stationary; never flew to windward; was stopped by an absence of wind at the core of the high-pressure systems; used leeward winds when flying away from the breeding grounds; either searched out favourable winds or tacked against lateral winds on returning to the colony (see the route north of Crozet); and covered 3,500 km just before its chick was hatched, a distance somewhere between the longer trips at the earlier stages of incubation and the much shorter ones typical of the brooding period (Table 1).



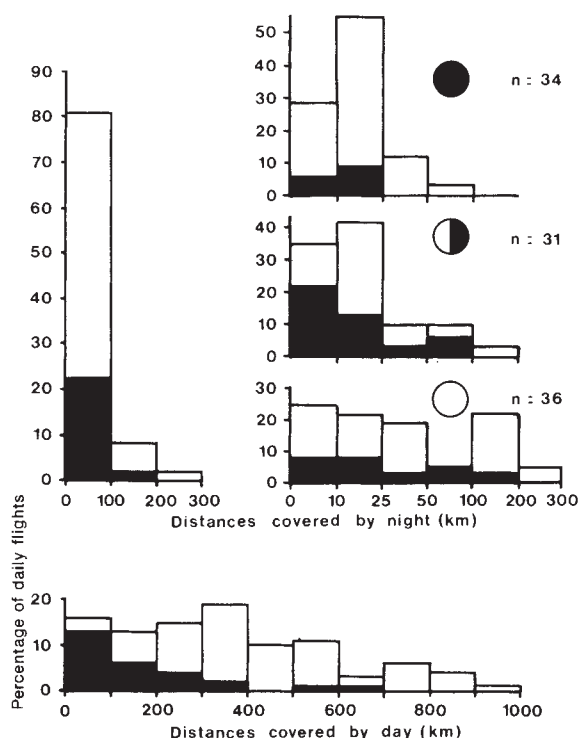


FIG. 3 In 24 h, Wandering albatrosses covered up to 936 km by day (bottom; $n=98$) plus up to 230 km by night (top; $n=101$). They did not fly so far during anticyclonic conditions (black). The longest night-time flights were undertaken when there was a full moon.

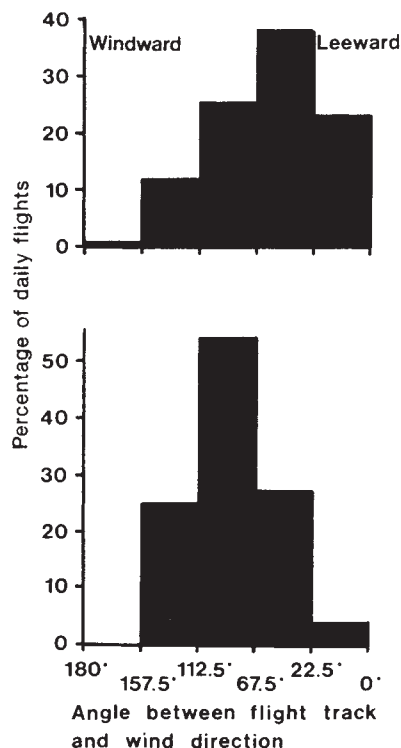


FIG. 4 Percentage of daily flights to windward and leeward. The birds almost never flew to windward (like sailing boats). They tended to use leeward winds during the outward journey (top; $n=157$) and lateral winds for the return to the nest (bottom; $n=178$).

strategy on use of the wind as the primary source of energy for their long foraging trips. The evolution and survival of the largest flying seabird has thus been possible only in the Austral Ocean. □

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Duration of sympatry and coevolution between the great spotted cuckoo and its magpie host

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PARASITIC cuckoos habitually lay their eggs in the nests of one or more host species. Because changes in one member of a host-parasite pair select for changes in the other member, it has been suggested that there is a coevolutionary arms race between cuckoos and their hosts^{1–6}. Comparisons of host species with species unsuitable as hosts, and the finding of host species from allopatric populations that tolerate nonmimetic eggs better than do species from sympatric populations, indicates that host behaviour has evolved in response to the behaviour of cuckoos⁵. Similarly, the observations that the laying procedure of cuckoos matches host defences², and that cuckoo egg mimicry parallels the discriminatory ability of the host species⁷, indicates that cuckoo behaviour has evolved in response to host counter-adaptations. Here we demonstrate that rejection of eggs of the parasitic great spotted cuckoo *Clamator glandarius* by the magpie *Pica pica* hosts varies between populations depending on cuckoo parasitism. In a population of ancient sympatry, magpies reject both nonmimetic and mimetic eggs; in an area of recent sympatry magpies show less rejection, particularly of mimetic eggs; in a population isolated from great spotted cuckoos, however, magpies show no rejection. Such differences in egg recognition among host populations affect the spatial population dynamics of parasites and their hosts. Parasitic cuckoos therefore are in a particularly favourable position in the coevolutionary arms race with their hosts in areas of recent sympatry.

In southern Europe, the great spotted cuckoo is a brood parasite on members of the corvid family, particularly the magpie⁸. A single cuckoo egg is laid quickly, usually during the laying period of the magpie host, while the male cuckoo distracts

the magpies from the nest^{8,9}. Great spotted cuckoo eggs are mimetic in size, coloration and spotting, and the laying of the thick-shelled egg from the rim of the nest usually causes one or several host eggs to become damaged^{8,9}. The great spotted cuckoo nestling hatches several days before the magpie nestlings, but does not eject host eggs or nestlings. Host reproductive success is markedly reduced by intense competition for food between the large fast-growing great spotted cuckoo young and the smaller magpie nestlings^{8,9}.

The great spotted cuckoo has increased its geographical range considerably during the twentieth century⁸, and it has therefore occurred together with its corvid hosts for different lengths of time. If coevolutionary arms races occur between the parasitic cuckoo and its hosts, we predict that in areas of recent sympatry with host populations, where hosts should be equipped with fewer counter-adaptations against the parasite, the parasitic cuckoo would have a greater reproductive success than it does in areas of ancient sympatry. We tested these predictions by studying interactions between great spotted cuckoos and their magpie hosts in Hoya de Guadix, southern Spain (37°18' N, 3°11' W), an area ~1,100 m above sea level that has been colonized by cuckoos since the early 1960s⁹; in Santa Fe, southern Spain (37°11' N, 3°43' W), an area of ancient sympatry⁹ at ~750 m above sea level ~60 km south-west of Guadix, and in Uppsala (59°51' N, 17°38' E), Sweden, where the great spotted cuckoo does not occur⁸.

We experimentally examined host responses to great spotted cuckoo eggs by introducing a painted mimetic model of the great spotted cuckoo egg, which has a light blue-green ground colour like magpie eggs, a quail *Coturnix coturnix* egg painted red, thereby altering the ground colour but not obscuring the markings of the egg, or both, into magpie nests during April–May 1989. Painted quail eggs differed markedly in appearance from mimetic models and real magpie eggs, both with respect to the ground colour and the markings, and were therefore highly nonmimetic (Fig. 1). To test whether magpies showed increased discriminatory ability in response to the simultaneous presence of two new eggs, one mimetic and one nonmimetic, we performed experiments with both a quail egg and a mimetic model. We recorded the removal of model eggs or quail eggs after 1, 2 and 5 days. The duration of the period until eggs were ejected was not related to the site of experiment, the colour of eggs or to their interaction (two-way analysis of variance, not significant). We recorded the stage in the host nesting cycle as egg-laying or incubation, depending on whether magpie eggs were laid after the start of the experiment. Time in the host nesting cycle did not affect the probability of an egg being ejected (log-likelihood ratio test¹⁰, $G^2 = 2.54$, degrees of freedom (d.f.) = 1, not significant).

Magpie responses to the introduction of both kinds of eggs differed significantly between sites. Mimetic eggs had the largest ejection rate of 78% ($N = 9$) in the Santa Fe area of ancient sympatry, with low values of 14% ($N = 14$) in the Guadix area of recent sympatry and 0% ($N = 12$) in the Uppsala area (Fig. 2; $G^2 = 18.93$, d.f. = 2, $P < 0.001$). Nonmimetic eggs had a large ejection rate of 100% ($N = 9$) in Santa Fe and 71% ($N = 14$) in Guadix, with a low rate of 0% ($N = 12$) in Uppsala (Fig. 2; $G^2 = 31.47$, d.f. = 2, $P < 0.001$).

Magpies also responded differentially to mimetic and nonmimetic eggs by rejecting a larger fraction of the nonmimetic quail eggs (Fig. 2; $G^2 = 14.72$, d.f. = 1, $P < 0.001$). Whereas the overall ejection rate in the area of ancient sympatry in Santa Fe was high, nonmimetic eggs tended to be ejected more often than mimetic eggs ($G^2 = 3.02$, d.f. = 1, $P = 0.07$). There was a highly significant difference in ejection rate between mimetic and nonmimetic eggs in the Guadix area of recent sympatry, where only 14% ($N = 14$) of mimetic eggs, but 71% ($N = 14$) of nonmimetic eggs were ejected ($G^2 = 10.01$, d.f. = 1, $P < 0.005$). Both mimetic and nonmimetic eggs were accepted by magpies in Uppsala, which is far outside the breeding range of the great

spotted cuckoo (Fig. 2). Differences in ejection rates of mimetic and nonmimetic eggs therefore were not due to differences in the material used for these two types of eggs. When we simultaneously introduced a mimetic and a nonmimetic egg into a magpie nest, ejection rates were not significantly different from those when only one egg was introduced at a time ($G^2 = 1.53$, d.f. = 1, not significant). But magpies usually ejected the nonmimetic egg earlier than the mimetic egg; in Santa Fe this occurred in all four cases of ejection, where only one egg was ejected, and in Guadix in all five cases of single egg ejection, differing significantly from a similar frequency of ejections of the two types of eggs (sign test, $P = 0.004$). Host discrimination against mimetic cuckoo eggs was therefore most pronounced in the area of ancient sympatry, whereas nonmimetic eggs were discriminated against to a large extent both in areas of recent and of ancient sympatry.

Great spotted cuckoos in the area of recent sympatry differed from older populations by expanding their host niche. First, they regularly parasitized three other corvid species in the Guadix area⁹, whereas the magpie was the only commonly parasitized species in nearby areas of ancient sympatry⁸, although all corvid species bred both in areas of recent and of ancient sympatry. Second, the number of great spotted cuckoos laying in each host nest was larger at Guadix than in other areas⁹. Third, individual female great spotted cuckoos frequently laid more than one egg in each host nest⁹. The three behavioural changes among great spotted cuckoos in the area of recent sympatry all led to increased reproductive success of the parasite⁹.

The experimental results could alternatively be due to gene flow between populations. Selection on magpies at Guadix against rejecting mimetic and for rejecting nonmimetic eggs in

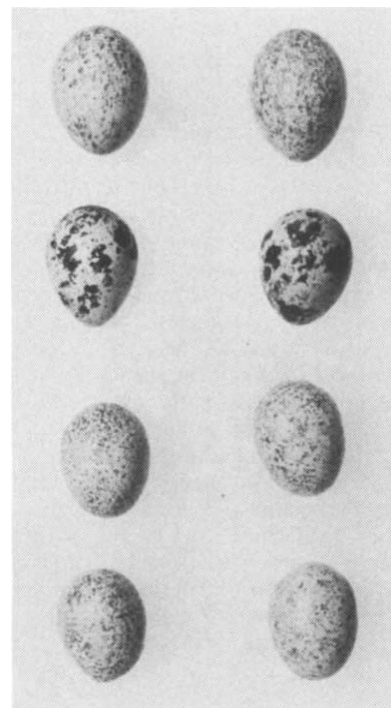


FIG. 1 Great spotted cuckoo eggs, mimetic model eggs, quail eggs (with the ground colour painted red) and magpie eggs. From the top downwards: great spotted cuckoo eggs, mimetic model eggs, quail eggs and magpie eggs. Model great spotted cuckoo eggs made out of plaster of Paris mixed with glue and painted with acrylic paints to resemble real great spotted cuckoo eggs were 30.8 mm × 23.1 mm and 9.2 g, similar to real fresh great spotted cuckoo eggs (31.2 mm × 23.4 mm and 9.5 g), to magpie eggs (32.6 mm × 23.5 mm and 9.0 g)⁹ and to quail eggs (32.5 mm × 25.1 mm and 8.5 g).

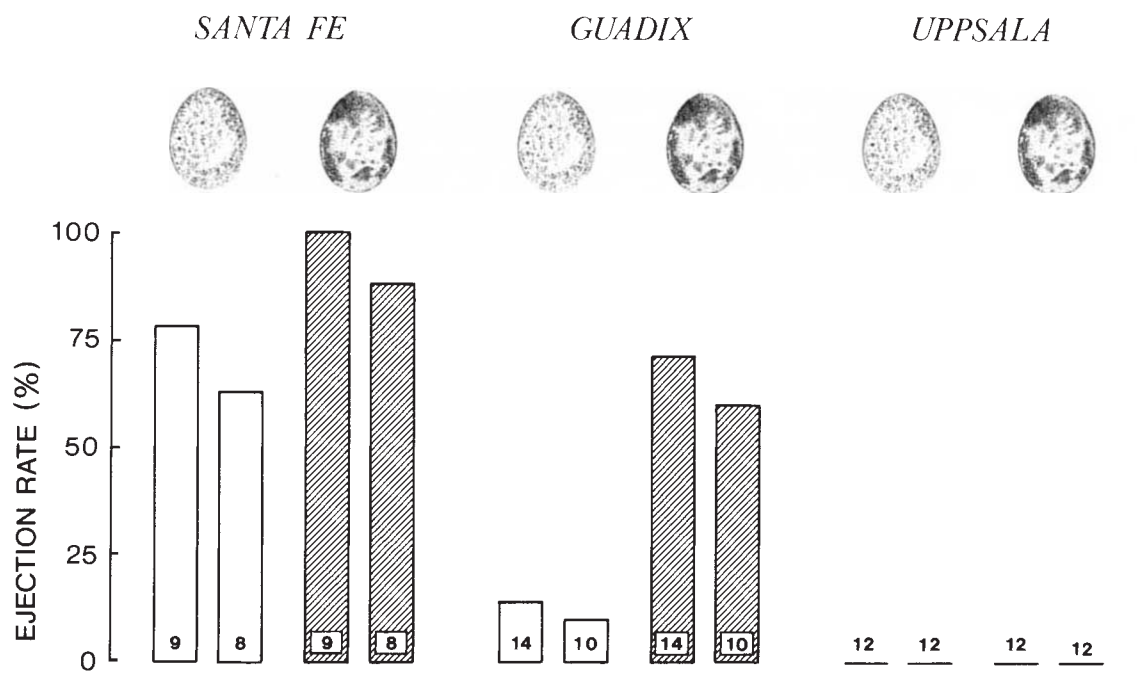


FIG. 2 Ejection rate (%) of mimetic and nonmimetic eggs from magpie nests in the Santa Fe area of ancient sympatry, the Guadix area of recent sympatry and the Uppsala area of allopatry. The two left columns for each study site represent ejection rates of mimetic eggs added singly (left column) or simultaneously with a nonmimetic egg (right column), whereas the two right columns for each study site represent ejection rates of nonmimetic eggs added singly (left column) or simultaneously with a mimetic egg (right column). The numbers of magpie nests used in the experiments are indicated. Mimetic eggs were made out of plaster of Paris mixed with glue and painted

with acrylic paints to resemble those of the great spotted cuckoo (left drawing of an egg for each study site), whereas nonmimetic eggs were real quail eggs with the ground colour painted red (right drawing of an egg for each study site). Magpie clutch size did not differ significantly between the three experimental groups in any of the study sites (one-way analysis of variance; $F=0.02$, $F=0.11$, $F=0.29$, respectively; all not significant). Egg laying was in progress in a similar proportion of nests when experiments were initiated in each of the three experimental groups in each area ($G^2=0.21$, $G^2=0.73$, $G^2=0.68$, respectively; d.f. = 2, all not significant).

the absence of great spotted cuckoos could explain the greater contrast between the rejection rates of mimetic and nonmimetic eggs at Guadix than at Santa Fe (Fig. 2), as well as the different behaviour of the distant Uppsala magpie population.

The behaviour of brood parasites in areas of recent sympatry with their hosts also has implications for the population dynamics of hosts and their parasites. The spread of genetically determined mimicry of parasite eggs through a population has been assumed to be very slow¹¹, because the proportion of hosts parasitized generally is small (at most a few per cent^{5,8}), but if this proportion is large, as for the magpie in the Guadix area (43% of 69 nests), then the spread of rejection behaviour will be more rapid. The population dynamics of brood parasites and their hosts, except temporal density dependence¹², should also be affected by the spatial variation in parasitism as described here (and in ref. 9). Because brood parasites are very successful in recently colonized areas, we hypothesize that the large number of range expansions among the relatively few species of brood parasites^{4,8,13-16} could be an important part of the spatio-temporal reproductive strategy of brood parasites. Parasite reproductive success is particularly high in areas of recent sympatry with their hosts, and recruitment of parasites from such recently colonized areas should further promote the expansion

of the brood parasite. Niche release of brood parasites and acquisition of new hosts particularly, should occur in areas of recent sympatry, where potential hosts have only recently been exposed to the selection pressures arising from parasites. □

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Calcium hotspots caused by L-channel clustering promote morphological changes in neuronal growth cones

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THE increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) that follows electrical activity in excitable cells influences various cellular functions. But the measured increase in average cytosolic $[\text{Ca}^{2+}]_i$ (typically 10 nM per action potential¹⁻³) is often less than the micromolar $[\text{Ca}^{2+}]_i$ required to activate proteins controlling cell function⁴⁻⁵. We now report that clustering of L-type Ca^{2+} channels causes $[\text{Ca}^{2+}]_i$ hotspots of average diameter 7 μm at the neuronal growth cone. At the hotspot, $[\text{Ca}^{2+}]_i$ changes of the order of 1 μM were recorded during 1-s voltage-clamp depolarizations, whereas a single action potential raised $[\text{Ca}^{2+}]_i$ by 89 nM. Depolarization will therefore activate enzymes with a micromolar requirement for Ca^{2+} at the hotspot. Local morphological changes near the site of the hotspot were induced by action potentials. We observed hotspots in all regions of the growth cone, usually at the base of processes extending from the growth-cone palm, but never in the neurite. The role of voltage-dependent Ca^{2+} influx in controlling nerve cell outgrowth has been a puzzle: although raised $[\text{Ca}^{2+}]_i$ triggers outgrowth of the growth cone margin, neurite elongation requires low $[\text{Ca}^{2+}]_i$ (refs 6-8). Our results resolve this paradox: electrical activity can selectively raise $[\text{Ca}^{2+}]_i$ in the growth cone, leaving neurite $[\text{Ca}^{2+}]_i$ low. Gross $[\text{Ca}^{2+}]_i$ gradients and localized hotspots have been previously reported in depolarized neurons^{3,9-12}. Patch-clamp, toxin-binding and freeze-fracture studies have demonstrated that calcium channels are grouped in clusters¹³⁻¹⁷. However, this is the first report that calcium channel clusters can cause $[\text{Ca}^{2+}]_i$ hotspots, and that channel clustering has a physiological role.

N1E-115 neuroblastoma cells express two types of Ca^{2+} current^{18,19} similar to the T- and L-type currents found in many neurons²⁰, although inactivation of the T-type current is slower than it is in other systems (Fig. 1). By using whole-cell patch-clamp of the cell soma, T- and L-type currents can be activated independently with the appropriate voltage protocol (Fig. 1a, b). This is possible because the voltage dependence of activation and inactivation is different for the two currents (Fig. 1g)^{19,21}. During activation of either current (Fig. 1c, d), growth cones $\leq 270 \mu\text{m}$ from the cell body were voltage-clamped (Fig. 1e, f). This allowed us to investigate the localization of these two types of channel on the growth cone by activating each current and measuring the spatial distribution of the $[\text{Ca}^{2+}]_i$ changes produced by digital imaging fluorescence microscopy. We illuminated growth cones with 380-nm ultraviolet light, and recorded the spatial distribution of light emitted by intracellular Fura-2 (I_{380}) with a silicon intensified target (SIT) camera and image processor.

During a 1-s voltage pulse that activated the L-type current, I_{380} recorded from the whole growth cone declined dramatically to $68 \pm 4\%$ (mean $\pm$ s.e.m.) of control, indicating a rise in $[\text{Ca}^{2+}]_i$ of $265 \pm 73 \text{ nM}$ ($n = 32$). A similar voltage protocol resulted in a $19 \pm 4\%$ increase of fluorescence at growth cones illuminated with ultraviolet light of 350-nm wavelength (four cells). These results indicate that the fluorescence changes were caused by a large increase in $[\text{Ca}^{2+}]_i$ at the growth cone (Fig. 2d), because artifacts such as a decline in the concentration of Fura-2, or a reduction of growth-cone thickness, would cause a fall of fluorescence at both excitation wavelengths. Several properties

of the growth-cone Fura-2 signal indicated that it was caused by Ca^{2+} influx through L-type channels. I_{380} declined throughout the 1-s depolarization, consistent with Ca^{2+} influx through a slowly inactivating channel (Fig. 2d). The voltage dependence and the divalent cation sensitivity (Fig. 3) of both the $[\text{Ca}^{2+}]_i$ change and the current were also consistent with L-type channel activation^{19,20}.

The changes in $[\text{Ca}^{2+}]_i$ at the growth cone during L-type current activation were highly localized to a few hotspots of

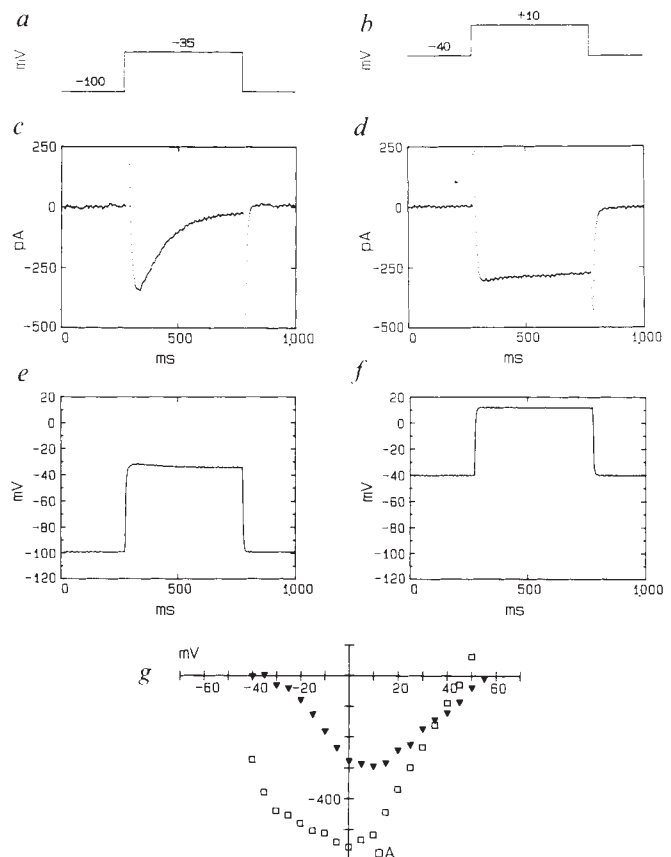


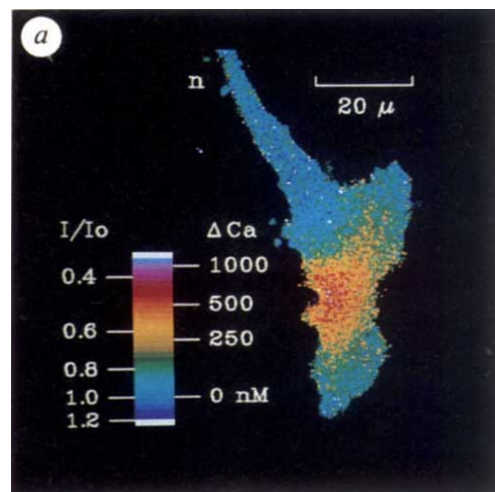
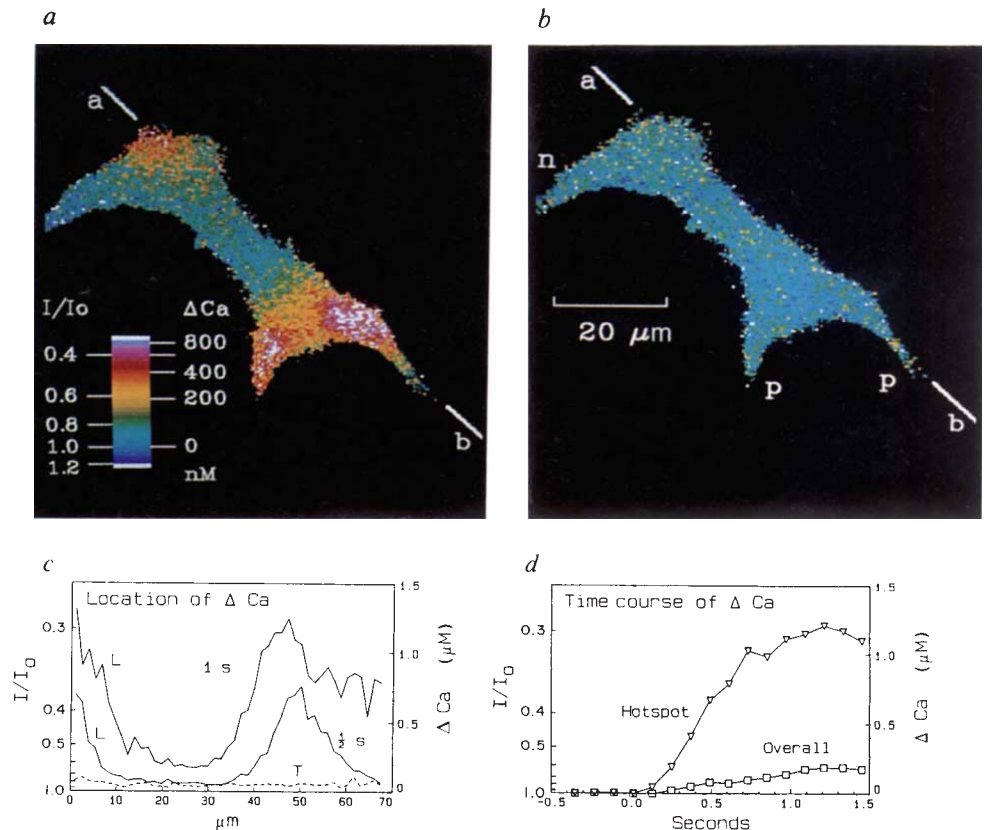
FIG. 1 Activation of T- and L-type Ca^{2+} currents by different voltage protocols applied by whole-cell voltage-clamp through a patch pipette on the cell body. **a**, Voltage protocol used to activate a T-type Ca^{2+} current. **b**, Protocol used to activate an L-type current. **c**, T-type current recorded through the voltage-clamping patch pipette on the cell body. The average rate of inactivation of the T-type current was $\tau = 83 \pm 7 \text{ ms}$ ($n = 23$). **d**, The L-type current showed little inactivation. The patch pipette contained an internal medium of 135 mM CsCl, 25 mM tetraethylammonium (TEA), 10 mM EGTA, 1 mM CaCl_2 , 1 mM MgCl_2 , 5 mM ATP, 0.1 mM leupeptin and 7.5 mM HEPES buffer, pH 7.1. Currents were corrected for membrane leakage. **e**, **f**, Membrane voltage at a growth cone 210 μm from the cell body during the depolarizing steps in **a** and **b** that elicited currents in **c** and **d**. Growth-cone membrane voltage was measured by whole-cell recording with a second patch pipette containing 130 mM KCl, 10 mM NaCl, 10 mM EGTA, 1 mM CaCl_2 , 1 mM MgCl_2 , 5 mM ATP, 0.1 mM leupeptin and 10 mM HEPES buffer, pH 7.1, connected to a high impedance amplifier. In all seven cells examined, growth cones $\leq 270 \mu\text{m}$ from the cell body were voltage-clamped to $\pm 2 \text{ mV}$ of the pipette voltage within 13 ms of a change of holding voltage. **g**, Current-voltage relation of leak-subtracted currents for the same cell, recorded 50 ms after the start of depolarization from holding voltages of -100 mV ($\square$) and -40 mV ($\blacktriangledown$). Temperature, 20°C . Because the L-type current is not activated at voltages more negative than -30 mV (Fig. 2), only T-type currents will be activated by the protocol shown in **a**. The T-type current is inactivated at a holding voltage of -40 mV (Fig. 1g); therefore only L-type currents will be activated by the protocol shown in **b**. Extracellular solution, 10 mM Ca^{2+} Tris-buffered saline¹⁸ with tetrodotoxin (TTX) (1 μM), CsCl (10 mM) and TEA (20 mM). Currents were eliminated either by the reduction of extracellular Ca^{2+} to 100 μM or by addition of CoCl_2 to 10 mM. This showed that they were Ca^{2+} currents. Their voltage dependence (Fig. 1g), was also consistent with L- and T-type Ca^{2+} currents.

FIG. 2 Calcium hotspots at the neuronal growth cone revealed by fluorescence imaging. *a* and *b*, Pseudocolour images of the growth cone indicating the spatial distribution of the change in $[Ca^{2+}]_i$ (ΔCa). *n*, Neurite leading to a cell body that lies out of the field of view; *p*, processes extending from the growth cone. The images were produced by dividing the background-corrected video frame that began 0.5 s after the start of depolarization by a background-corrected video frame acquired at the holding voltage 0.5 s before the start of depolarization. The image in *a* was produced by activation of L-type Ca^{2+} channels during a 1-s voltage pulse to +10 mV from a holding voltage of -40 mV. The increase in $[Ca^{2+}]_i$ was highly localized in three discrete hotspots. The image in *b* was produced by activation of T-type Ca^{2+} channels during a voltage pulse to -35 mV from a holding voltage of -100 mV. The increase in $[Ca^{2+}]_i$ was 82 nM and uniform. A detectable $[Ca^{2+}]_i$ increase (of 28 ± 8 nM) was observed during T-type current activation in nine growth cones. In all cases, hotspots were not observed, although gross gradients in which the calcium change was greatest at the growth cone periphery were sometimes seen (Fig. 3c). These small nonlocalized gradients were probably caused by gradients of surface-area/volume ratio; that is, the growth cone is thinnest at the periphery. *c*, Spatial distribution of Ca^{2+} during activation of T- and L-type channels. Each distribution was calculated from the values of I_{380} along the axis *a-b* indicated in the images in *a* and *b*. Dashed line, $[Ca^{2+}]_i$ increase produced by activation of T-type channels, calculated from the image in *b*. Solid lines, $[Ca^{2+}]_i$ increase 0.5 s (calculated from the image in *a*) and 1 s after activation of L-type channels. *d*, Time course of $[Ca^{2+}]_i$ changes during 1-s activation of L-type channels. $\square$, $[Ca^{2+}]_i$ calculated from I_{380} of whole growth cone; ∇ , $[Ca^{2+}]_i$ at the lower hotspot on the *a-b* axis. Temporal resolution is limited by the time constant of the SIT tube (150 ms). Subtracted backgrounds were either images acquired before Fura-2 introduction or uniform fields corresponding to the fluorescence of the coverslip. Both methods gave the same result, because growth-cone autofluorescence

mean diameter $7.3 \pm 0.4 \mu\text{m}$ ($n = 44$; Fig. 2a). The horizontal resolution of the microscope is $\sim 7 \mu\text{m}$, therefore the true hotspot diameter is likely to be smaller than this value. In one hotspot, Fura-2 became saturated with Ca^{2+} before the end of the 1-s depolarization, implying that the $[Ca^{2+}]_i$ was $> 4 \mu\text{M}$; in 34 other hotspots, $[Ca^{2+}]_i$ increased by $1.0 \pm 0.2 \mu\text{M}$. We estimated the concentration of Fura-2 in the growth cone to be $\sim 100 \mu\text{M}$ ⁸. Fura-2 at this concentration does not change either the resting cell $[Ca^{2+}]_i$ or the growth-cone behaviour⁸, but the Ca^{2+} buffering capacity of the cytosol is likely to have been increased significantly. Under normal conditions, therefore, $[Ca^{2+}]_i$ changes at the hotspot could be even larger than we measured. The concentration profile across the hotspot broadened as depolarization was maintained (Fig. 2c), indicating that the principal mechanism by which $[Ca^{2+}]_i$ increased in regions outside the hotspot was diffusion of Ca^{2+} from the hotspot. Growth cones contained between 1 and 5 hotspots (mean, 1.9 ± 0.2 ; $n = 32$).

A uniform distribution of Ca^{2+} channels within the cell membrane could produce $[Ca^{2+}]_i$ hotspots by three mechanisms. First, hotspots could represent areas where Ca^{2+} buffering capacity is unusually low. Second, hotspots could lie under invaginations of the cell membrane that produce a locally high surface-area/volume ratio. We did not observe $[Ca^{2+}]_i$ hotspots during activation of T-type channels, inconsistent with these

was negligible. T-type and L-type currents were elicited between 5 and 10 min after rupturing the patch. I_{380} did not change during controls in which the growth cone was not depolarized, indicating that dye bleaching was negligible. Extracellular and pipette solutions as described in Fig. 1, except that the pipette solution contained 5 mM Fura-2 without $CaCl_2$ or EGTA. Temperature, 20 °C. Unstimulated growth-cone $[Ca^{2+}]_i$ was measured using ratio imaging and found to be a uniform 95 ± 20 nM ($n = 22$). The $[Ca^{2+}]_i$ change from this resting level was calculated from the fractional change of I_{380} using the equation $[Ca^{2+}]_i = K_d (I_{380}^0 - I_{380}) / (I_{380} - I_{380}^{\text{sat}})$, where K_d the dissociation constant ($0.135 \mu\text{M}$ at 20 °C)²⁶, I_{380}^0 is the fluorescence intensity at zero $[Ca^{2+}]_i$, I_{380} is the measured fluorescence intensity, and I_{380}^{sat} is the fluorescence intensity at saturating $[Ca^{2+}]_i$, measured *in situ* as $0.102 I_{380}^0$.



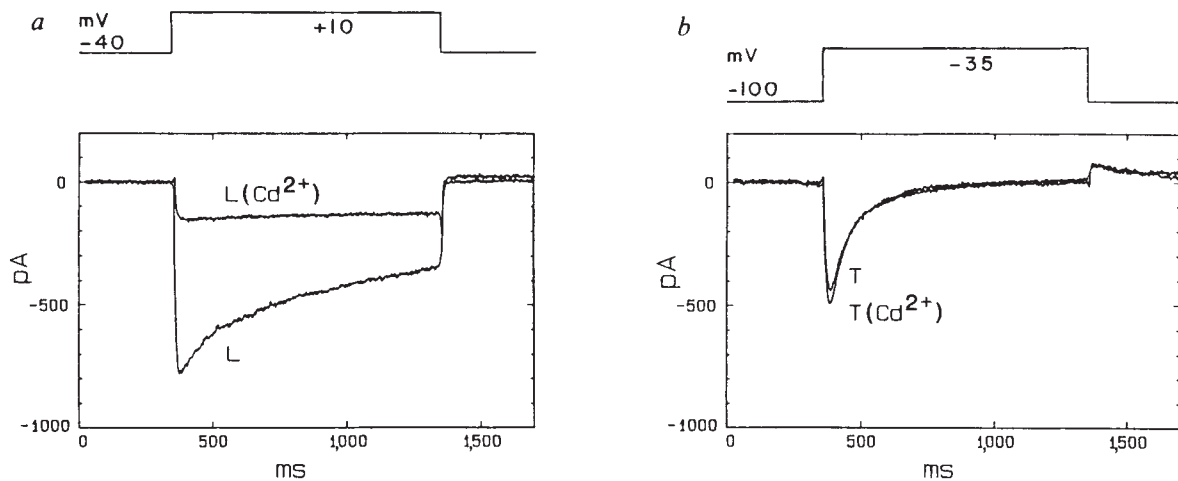
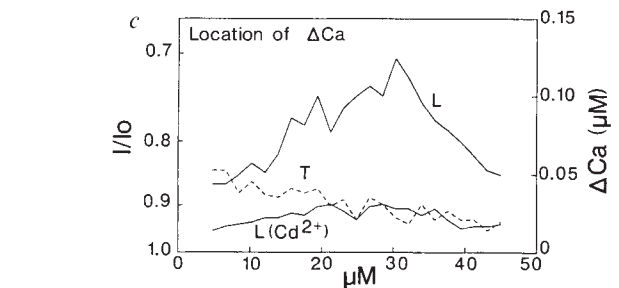


FIG. 3 Selective Cd^{2+} block of L-type current and Ca^{2+} hotspot. The relatively uniform $[\text{Ca}^{2+}]_i$ change observed during activation of the T-type current (Fig. 2b, c) does not rule out the possibility that the hotspots seen during L-type current activation are caused by an inhomogeneity of Ca^{2+} buffering that only appears at high $[\text{Ca}^{2+}]_i$ levels. To test this possibility, we used Cd^{2+} to selectively reduce L-type currents. When the $[\text{Ca}^{2+}]_i$ changes during L- and T-type current activation were similar, hotspots persisted during L-type current activation, ruling out a buffering explanation. *a*, Membrane voltage and leak-subtracted whole-cell current during activation of L-type channels. L, control; L(Cd^{2+}), in presence of $70 \mu\text{M}$ CdCl_2 , which reversibly reduced L-type currents by $72 \pm 5\%$ ($n=8$); *b*, Membrane voltage and membrane leak-subtracted whole-cell current during activation of T-type channels. T, control; T(Cd^{2+}), in the presence of $70 \mu\text{M}$ CdCl_2 . T-type currents were not significantly reduced (% change = $+5 \pm 8$, $n=4$). L-type currents were much more sensitive to Cd^{2+} than were T-type currents, as described for this and other preparations^{19,20}. *c*, Spatial distribution of Ca^{2+} during activation of T- and L-type channels along a line through the centre of a hotspot. T, 0.5 s after activation of T-type current; L, 0.5 s after activation of L-type current. The hotspot $[\text{Ca}^{2+}]_i$ change in this growth cone was small, facilitating comparison of $[\text{Ca}^{2+}]_i$ changes during activation of L- and T-type currents. L(Cd^{2+}), 0.5 s after activation of L-type current in medium containing $70 \mu\text{M}$ CdCl_2 . Cd^{2+} reduced the $[\text{Ca}^{2+}]_i$ change during L-type current activation by



$70 \pm 11\%$ ($n=7$). The similar Cd^{2+} sensitivity of whole-cell L-type current and growth-cone $[\text{Ca}^{2+}]_i$ change confirm that L-type channels are responsible for the Ca^{2+} influx at the hotspot. Although Cd^{2+} is unlikely to enter the growth cone during L-type current activation²⁷, any Cd^{2+} that does enter will produce a fluorescence signal similar to Ca^{2+} . To avoid possible problems due to irreversible Cd^{2+} binding to Fura-2, we did not make any further measurements on cells after the first depolarization in medium containing CdCl_2 . Extracellular application of the Ca^{2+} channel blocker CoCl_2 (10 mM) eliminated both the L- and T-type currents and the associated I_{380} changes (seven cells).

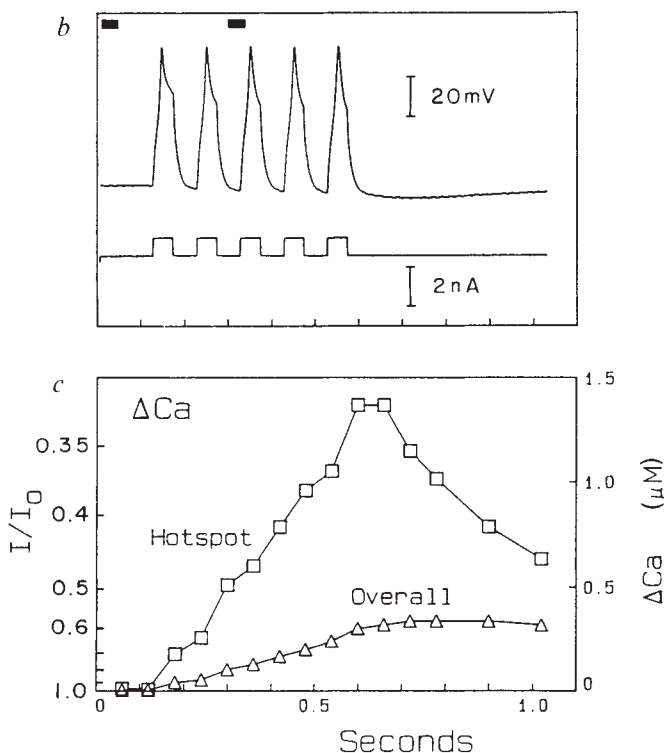


FIG. 4 Ca^{2+} hotspots observed under physiological conditions. *a*, Pseudocolour image of a growth cone indicating the spatial distribution of the increase in $[\text{Ca}^{2+}]_i$ that occurred during two action potentials. This was produced by dividing images acquired at the times indicated by horizontal bars in Fig. 4b. *b*, Record of membrane voltage (upper trace) during a train of five action potentials elicited by depolarizing current pulses (lower trace). *c*, Time course of $[\text{Ca}^{2+}]_i$ changes during the action potential train. Δ , $[\text{Ca}^{2+}]_i$ calculated from I_{380} of whole growth cone. $\square$, $[\text{Ca}^{2+}]_i$ at the hotspot. Extracellular solution, 1.8 mM CaCl_2 Tris-buffered saline¹⁸. Temperature, $32-36^\circ\text{C}$. The micropipette contained 5 mM Fura-2, 140 mM KCl, 5 mM HEPES buffer, pH 7.1. The recorded holding voltage was -67 mV , but micropipettes with this filling solution exhibit unpredictable changes of tip potential on cell impalement. $[\text{Ca}^{2+}]_i$ change calculated as in Fig. 2, except that measured resting $[\text{Ca}^{2+}]_i$ was $107 \pm 11 \text{ nM}$ ($n=53$), $K_d=0.224 \mu\text{M}^{26}$, and $I_{380}^{\text{sat}}=0.119/0.380$. To examine a possible correlation between the location of hotspots and the location at which the growth cone margin grew out, we identified in each growth cone the site of maximum margin outgrowth. We scored a hotspot as close to the site of maximum outgrowth if lines from the centre of the growth cone to the site of maximum outgrowth and the hotspot diverged by $\leq 10^\circ$. The seven growth cones showing outgrowth in the 4 min after action potential stimulation contained a total of 12 hotspots. In five growth cones, a hotspot was close to the site of maximum outgrowth. By chance, we would expect only 1/18th of all hotspots to lie in a particular 20° sector, assuming two-dimensional geometry. Hotspots and margin advance were therefore highly correlated ($\chi^2=30.2$, $P<0.001$). N1E-115 cells were cultured in DMEM supplemented with 6% FCS. Cells growing on polylysine-coated coverslips were induced to differentiate by the addition of either 2% dimethylsulphoxide, this experiment, or 0.5 mM dibutyl cyclic AMP (diBcAMP) and used 2-9 days later. Hotspot characteristics were independent of the agent (dimethylsulphoxide, 16 cells; diBcAMP, 11 cells) used to induce differentiation of the cells.

mechanisms (Fig. 2b, c; Fig. 3c). Third, as suggested for adrenal chromaffin cells²², Ca^{2+} influx could activate release of Ca^{2+} from internal stores that are distributed non-uniformly throughout the cytoplasm. But Cd^{2+} , a specific blocker of the L-type calcium current^{19,20}, reduced the L-type current and the change in $[\text{Ca}^{2+}]_i$ by the same extent (Fig. 3), inconsistent with this hypothesis. Thus it is highly likely that the hotspots we observed resulted from nonuniform clustering of L-type channels within the growth-cone membrane.

Although many studies have indicated a role for $[\text{Ca}^{2+}]_i$ in modulating growth-cone behaviour, the results are paradoxical. An increase in $[\text{Ca}^{2+}]_i$ can cause outgrowth of the growth-cone margin^{7,8}, but it also inhibits neurite elongation^{6,8}. Consistent with the hypothesis that $[\text{Ca}^{2+}]_i$ promotes localized margin outgrowth, we found that hotspots were usually located at the base of processes that had extended from the growth cone (for example, structures marked p in Fig. 2b). Out of 32 growth cones, 24 had processes. In these 24 growth cones, process bases comprised only 20% of the total circumference of the growth cone (measured as angles subtended at the growth-cone centre), but 38 hotspots out of a total of 47 were located at the process base, indicating a high spatial correlation ($P < 0.001$, χ^2 test). We observed hotspots in both proximal and distal regions of the growth cone. No hotspots were observed in the neurite (marked n in Figs 2b, and 4a), where raised $[\text{Ca}^{2+}]_i$ inhibits growth.

To examine the $[\text{Ca}^{2+}]_i$ changes produced under physiological conditions, we used a conventional microelectrode to pressure-inject Fura-2 and electrically stimulate cells (Fig. 4b) maintained in a medium containing 1.8 mM Ca^{2+} at 32–36 °C. Distinct Ca^{2+} hotspots could be detected during a train of five action potentials at 10 Hz (Fig. 4a). In two growth cones, hotspot $[\text{Ca}^{2+}]_i$ increased to $>1 \mu\text{M}$ during the train (for example, Fig. 4c). On average, hotspot $[\text{Ca}^{2+}]_i$ increased by $89 \pm 26 \text{ nM}$ per action potential ($n = 11$); that is, 12 action potentials would be required to raise hotspot $[\text{Ca}^{2+}]_i$ to micromolar levels. In 7 of the 11 growth cones, we saw outgrowth of the growth-cone margin by $6 \pm 1 \mu\text{m}$ in the 4 min following action-potential stimulation. In five of these, the site at which the greatest outgrowth occurred was close to a hotspot, indicating a strong spatial correlation between hotspots and site of outgrowth ($P < 0.001$, χ^2 test; see Fig. 4 legend for details). $[\text{Ca}^{2+}]_i$ is thought to affect cell morphology by activating proteins that cut, nucleate and cap actin filaments^{5,23}. These proteins are activated by micromolar concentrations of $[\text{Ca}^{2+}]_i$. Our results demonstrate that at the hotspot, relatively few action potentials will raise $[\text{Ca}^{2+}]_i$ sufficiently to activate these proteins.

The results described here could explain how the pattern of connections formed in the developing nervous system depends on the electrical activity in the neurons²⁴. Unstimulated growth cones tend to grow straight forwards²⁵. But hotspots, and therefore the margin outgrowth caused by electrical activity, are asymmetric. $[\text{Ca}^{2+}]_i$ hotspots produced by clustering of L-type Ca^{2+} channels therefore provide a mechanism for triggering the turning of electrically active neurites, and could have a crucial role during development.

Note added in proof. Consistent with the identification of the hotspot calcium channels as L-type and not N-type, the dihydropyridine agonist BAY K8644 ($1 \mu\text{M}$) enhanced the L-type current by $170 \pm 14\%$ ($n = 8$), but had no effect on the T-type current.

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Homing of a $\gamma\delta$ thymocyte subset with homogeneous T-cell receptors to mucosal epithelia

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IN mice $\gamma\delta$ T-cell populations with distinct T-cell receptor (TCR) repertoires and homing properties have been identified. Diversified populations are found in lymphoid organs^{1–3} and intestinal epithelia^{4,5}. By contrast, the $\gamma\delta$ T-cells that have been found in the murine skin are homogeneous. They express a TCR consisting of one particular V γ 5 and one particular V δ 1 chain^{5,6} and seem to originate from early fetal thymocytes^{6,7}. We have now systematically analysed many tissues by immunohistochemistry and TCR gene sequencing aided by the polymerase chain reaction. These studies revealed a second homogeneous $\gamma\delta$ T-cell subset in epithelia not of the intestine and skin, but of the vagina, uterus and tongue. The TCR expressed by this $\gamma\delta$ T-cell subset consists of the same V δ 1 chain. Cells that express this particular TCR have previously been shown to be positively selected in the late fetal thymus⁷.

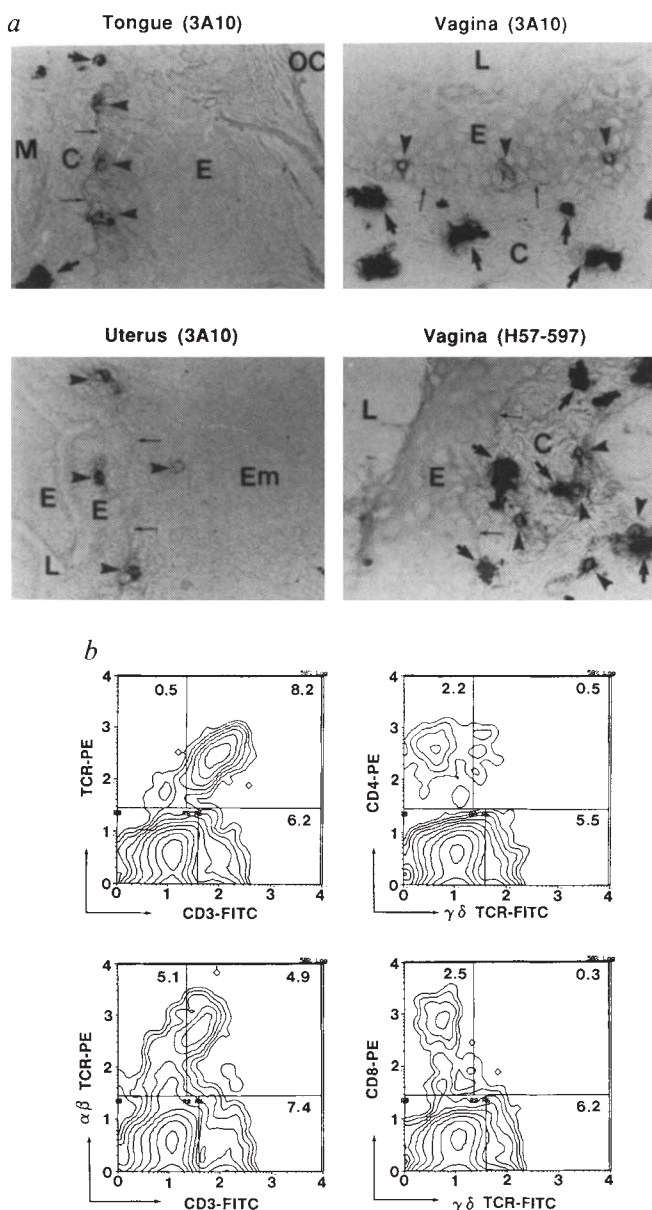
We performed immunohistological analysis using monoclonal antibody 3A10 (ref. 8) on frozen tissue sections of various organs from 8–10-week-old BALB/c mice. We found $\gamma\delta$ T cells in most of the tissues analysed, frequently in close association with epithelial cells (Table 1). Representative data of tongue, vagina and uterus, which are discussed here, are shown in Fig. 1a. Most $\gamma\delta$ T cells in tongue and vagina were attached to, or embedded in, the basal layer of stratified squamous epithelium (Fig. 1a). About 30% of $\gamma\delta$ T cells in uterus were associated with simple columnar luminal epithelium (Fig. 1a). The rest of uterine $\gamma\delta$ T cells were broadly distributed in endometrium and myometrium (Fig. 1a, and data not shown). Staining of adjacent sections with monoclonal antibody H57-597 (ref. 9) showed that some $\alpha\beta$ T cells were also present in most tissues, but they were mainly distributed in subepithelial regions (Fig. 1a, and data not shown). $\gamma\delta$ T cells seemed to be localized to all epithelia that faced internal or external body surfaces, but not to epithelia within organs such as kidney, pancreas, liver or testis (Table 1).

To determine the use of V γ gene segments, we extracted DNA from lymphocyte preparations of various tissues of C57BL/6 mice (8–10-weeks old) and amplified it by the polymerase chain

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reaction (PCR)¹⁰ using primers for *J* γ 1 and various *V* γ -segments. We detected the amplified DNA by Southern-blot analysis using a *J* γ 1-specific oligonucleotide probe (Fig. 2a). We determined the relative intensity of the bands by using a Fuji Bioimage Analyser and converted this to the relative frequency of the corresponding rearrangement in a given DNA sample by using the standardization curves shown in Fig. 2b. As expected from previous studies, *V* γ 4, *V* γ 5 and *V* γ 7 were preferentially used by $\gamma\delta$ T cells in peripheral lymphoid organs, skin and small intestine, respectively (Fig. 2a)⁴⁻⁶. It was of interest that *V* γ 6-*J* γ 1 rearrangements, which predominate in the fetal and newborn thymocyte preparations (Fig. 2a, compare the ratio of *V* γ 4 and *V* γ 6 bands of fetal and adult thymus)^{7,11,12} predominated in lymphocyte preparations from the tongue, uterus and vagina, although some *V* γ 4-*J* γ 1 rearrangements, and very few *V* γ 7-*J* γ 1, *V* γ 5-*J* γ 1 (Fig. 2a) and *V* γ 1-*J* γ 4 rearrangements (data not shown), were also observed in these T cells. Flow cytometric analysis of lymphoid cell suspension from these tissues showed that about half of the cells carrying the CD3 antigen were $\gamma\delta^+$ TCR, Thy-1⁺, CD4⁺ and CD8⁺ (Fig. 1b). Few if any of these cells could have been derived from 'contaminating' blood, because only 3% of CD3⁺ blood cells express $\gamma\delta$ TCRs⁸.

TABLE 1 Distribution of $\gamma\delta$ T cells in mouse

Organs	Presence of $\gamma\delta$ T cells*	Contact with epithelial cells	No. of cells per animal†
Digestive system			
Tongue	+	+	
Oesophagus	+/- ‡	+	
Stomach	+	+	
Small intestine	+	+	1×10^6
Large intestine	+	+	1×10^5
Liver	-		
Pancreas	-		
Reproductive system			
Ovary	+/-		
Uterus	+	+	1×10^5
Vagina	+	+	1×10^5
Testis	-		
Epididymis	+/-	-	
Urinary system			
Kidney	-		
Bladder	+	+	
Others			
Skin	+	+	5×10^6
Brain	-		
Heart	-		
Lymphoid organs			
Thymus	+		1×10^6
Spleen	+		8×10^5
Lymph-node	+		1×10^5
Blood	+		6×10^4

* The data from BALB/c mice examined by immunohistological experiments except for blood.

† The numbers of $\gamma\delta$ T cells in lymphoid organs (C57BL/6) were determined by flow cytometry⁸. For other organs, the approximate numbers of $\gamma\delta$ T cells were estimated as follows: (number of $\gamma\delta$ T cells per cross section of the organ) $\times$ ((length of the organ in μ m)/(thickness of the section (6 μ m) $\times$ 2)). The calculation is based on the assumption that a stained T cell (~8 μ m in diameter) observed would almost always occupy two adjacent tissue sections (each 6 μ m thick). We also assume that the tissues or organs examined have a constant diameter.

‡ Very scarce distribution of $\gamma\delta$ T cells is indicated by +/-.

FIG. 1 Demonstration of $\gamma\delta$ T cells in tongue, vagina and uterus. **a**, Immunohistostaining of tissue sections. Fresh tissues from 8-to-10-week-old BALB/c mice were snap-frozen, and 6 μ m sections were fixed with cold acetone, stained with a monoclonal antibody anti- $\gamma\delta$ TCR (3A10), or a monoclonal antibody anti- $\alpha\beta$ TCR (H57-597), affinity-purified goat anti-hamster IgG-biotin (Caltag, California), and avidin-horseradish peroxidase conjugates. The specificity of the staining was confirmed by the control staining of adjacent sections without the primary antibody. Arrowheads indicate some of the stained TCR-positive cells. Large and small arrows indicate endogenous peroxidase activity and basal lamina, respectively. E, epithelium; OC, oral cavity; L, lumen; M, striated voluntary muscle; C, connective tissue; Em, endometrium. **b**, Flow cytometric analysis of lymphocytes prepared from reproductive organs. Conjugates of anti-CD3(145-2C11)-fluorescein isothiocyanate (FITC), anti- $\gamma\delta$ TCR(3A10)-biotin or -FITC, anti- $\alpha\beta$ TCR(H57-597)-biotin, anti-CD4(GK1.5)-phycoerythrin (PE), anti-CD8(53-6.7)-biotin, and streptavidin-PE. Quadrants were determined on the basis of the control staining with streptavidin-PE and a goat anti-hamster-FITC conjugate. The percentage of positive cells in each quadrant is shown. Fluorescence intensity is shown in log₁₀ scale.

METHODS. Vaginae and uteri from 10-week-old C57BL/6J mice were extensively washed with PBS, minced into ~1-mm³ pieces, and digested for 1 h with 0.5% trypsin and 0.02% EDTA in PBS. Lymphoid cells were pelleted by centrifugation through 30% Percoll (Pharmacia LKB, Sweden) at 1,750 r.p.m. for 10 min, washed, and incubated overnight at 37 °C in RPMI 1640 medium containing 10% FCS and lymphokines (interleukins-1 and -2). Nonadherent cells were collected and examined by procedures described previously⁸.

We cloned many *V* γ 6-*J* γ 1 PCR products from these cells and determined the junctional sequences of randomly chosen clones. Most of these sequences were in-frame and identical with the canonical γ -chain gene sequence expressed by late fetal $\gamma\delta$ thymocytes⁷ (Fig. 2c). To confirm the identity of TCR expressed by the uterus- and vagina-associated $\gamma\delta$ T-cell subset with the late fetal $\gamma\delta$ thymocytes, we further cloned and sequen-

ced *V* δ 1-*J* δ 2 PCR products prepared from the DNA isolated from this peripheral $\gamma\delta$ T-cell subset. Indeed, we found that the *V* δ 1-*D* δ 2-*J* δ 2 sequence was identical to that used in the thymocytes (Fig. 2d)⁷. By contrast, most other sequenced *V* γ -*J* γ junctions in the same DNA preparations were out-of-frame. Therefore, all of four *V* γ 5-*J* γ 1 sequences were out of frame, and only four of twenty *V* γ 4-*J* γ 1 sequences were in-frame, and

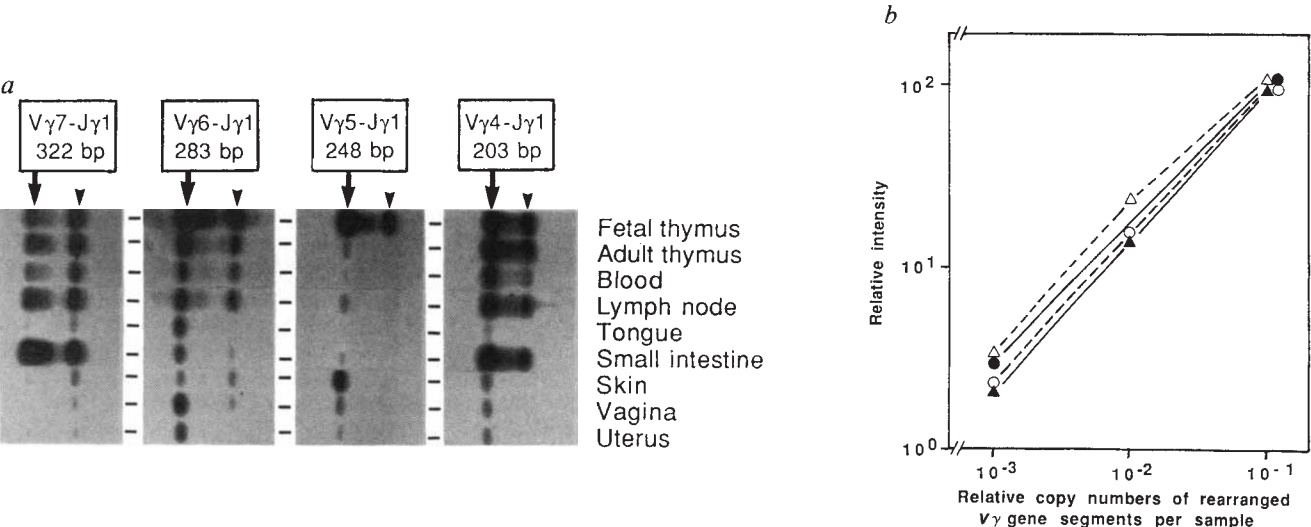


FIG. 2 a, Detection of rearranged TCR γ -chain gene segments in various organs by Southern-blot analysis of PCR-amplified products of chromosomal DNA. The blots of PCR products were probed with a *J* γ 1-specific oligonucleotide probe. The expected length of the respective PCR product from previously characterized $\gamma\delta$ T-cell hybridomas (V4, KN6; V5, KI129; V6, KI21; and V7, KN106)^{3,12} is indicated by the arrows. The arrowheads indicate dimeric products which could have formed using the imperfect inverted repeats within the *J* γ -gene segment. b, Standardization curves of PCR reactions. The control experiments using serial dilutions of DNA from the hybridomas indicated that the intensity of PCR bands is proportional to the copy number of the corresponding genes. $\circ$, *V* γ 4-*J* γ 1; $\bullet$, *V* γ 5-*J* γ 1; Δ , *V* γ 6-*J* γ 1; $\blacktriangle$, *V* γ 7-*J* γ 1. c, *V* γ -*J* γ 1 junctional sequences in female reproductive organs (uterus and vagina) and tongue. d, *V* δ 1-*D* δ 2-*J* δ 2 junctional sequences are aligned with germline sequences of *V* γ and *J* γ 1^{4,7,11}, *D* δ 2 and *J* δ 2¹⁹ gene segments. *V*-*J* or *V*-*D*-*J* joining can be either compatible or incompatible with the translation of the *J* segment in a required frame. This is indicated for each junctional sequence. The sequences indicated by asterisks are identical with the canonical sequence observed in the *V* γ 6 subset of fetal thymocytes⁷. The in-frame *V* γ 4 clone indicated by parenthesis cannot encode a complete γ -chain, because of the termination codon (TAA, underlined) present in the *V* γ 4 gene segment. Also shown is the frequency of DNA clones obtained for each junctional sequence.

METHODS. Cells were prepared from the lymphoid organs, skin and intestines as previously described^{4,5}. Cells from the other epithelia were prepared by digestion with trypsin followed by centrifugation as described in the legend to Fig. 1. These samples still contain large numbers of nonlymphoid cells. High relative molecular mass DNA was extracted, digested with *Eco*RI, and subjected to PCR. The PCR primers used were: *V* γ 4, 5'-TGTCCTTGCAACCCCTACCC-3'; *V* γ 5, 5'-TGTGCACTGGTACCAACTGA-3'; *V* γ 6, 5'-AGTGTTCAGAACCCGATGC-3'; *V* γ 7, 5'-AAGCTAGAGGGTCTCTGTC-3'; and *J* γ 1, 5'-CAGAGGGAATTACTATGAGC-3'; *V* δ 1, 5'-GAATGGAAC-TAATGCTCTGT-3'; and *J* δ 2, 5'-CAACTTACGGGCTCCAC-3'. Test DNA (2 μ g) was made up to 100 μ l in a reaction mixture (10 mM Tris-HCl buffer, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 100 pmol both primers, 0.25 mM each dNTP, 0.01% gelatin, and 2.5 U *Taq* polymerase). Thirty PCR cycles were run with 1 min at 92 $^{\circ}$ C, 2 min at 50 $^{\circ}$ C for γ -chain genes, or at 45 $^{\circ}$ C for δ -chain

genes, and 3 min at 72 $^{\circ}$ C per cycle. The PCR products were analysed by the Southern-blot hybridization technique previously described⁴. A *J* γ 1 oligonucleotide, 5'-TGAATTCCTCTGCAATACCTTG-3', end-labelled with ³²P was used as a probe. Autoradiograms were generated using the FUJI BA100 Bio-image analyzer (FUJI Photo Film Co. Ltd.). To make the standardization curves, the hybridoma DNAs were serially diluted with a DNA solution (2 μ g per 100 μ l) derived from the PCC3 embryonal carcinoma cell line²⁰ lacking any TCR γ -gene rearrangement, and were subjected to PCR reaction and Southern-blot hybridization. The intensities of autoradiographic bands were quantitated using the Fuji Bioimage Analyzer, and were plotted on a log scale. We estimated copy numbers of rearranged *V* γ gene segments in a given test sample from the standardization curves. The preference of a particular *V* γ -*J* γ 1 rearrangement was estimated from the ratio of copy numbers of rearranged *V* γ -*J* γ 1 segments for each sample. The PCR products were purified, cloned and sequenced according to the procedure described elsewhere⁷.

one of these contained a premature termination codon in the 3' end of the V γ 4 segment (Fig. 2c). V γ 6 rearrangements also occur in many other tissues (Fig. 2a). But in all cases that have been studied in detail, the junctional sequences are different from the canonical sequence. For instance, 12 V γ 6-J γ 1 junctions from lymph node cells (this study, data not shown) and 8 V γ 6-J γ 1 junctions from intestinal epithelial lymphocytes (ref. 5; V γ 4-J γ 1 in the nomenclature used therein) were diverse, and none was identical with the canonical sequence. Therefore, homing to epithelia of vagina, uterus and tongue does not seem to be a property of all cells expressing V γ 6, but a property of cells expressing the canonical V γ 6-V δ 1 sequences.

The study described here shows that in mice, many peripheral $\gamma\delta$ T cells are localized in epithelia not only of the skin and the intestine, but also of several other mucosae that face body surfaces. These observations support the view that $\gamma\delta$ T cells play a part in the surveillance of body surfaces that are exposed to environmental hazards^{13,14}. It is of interest that the TCR repertoires of $\gamma\delta$ T cells differ in various tissues. Most intriguingly, two epithelium-associated $\gamma\delta$ T-cell subsets have distinct and homogeneous subsets of TCR, one in the skin^{5,6} and, as shown here, one in mucosal epithelia or organs such as vagina, uterus and tongue. Both subsets seem to originate from positive cellular selection in the fetal thymus⁷. The homing of these $\gamma\delta$ T-cells to epithelial sites could be mediated by TCR. But this possibility is neither very attractive nor consistent with the epithelial homing of $\gamma\delta$ T-cells with 'wrong receptors' observed in transgenic mice¹⁵. It is also unlikely that the localization of a given $\gamma\delta$ T-cell subset results from its specific retention in certain epithelia rather than by specific homing¹⁵. It is more likely that the two monospecific cell populations represent distinct $\gamma\delta$ T-cell sublineages differing not only in their TCR, but also in homing receptors and possibly other properties. One or both sublineages could be absent, or have different properties in other species such as man, in which a close association of $\gamma\delta$ T-cells with epidermal and mucosal epithelial cells has not been observed¹⁶⁻¹⁸. Each of the monospecific subset of $\gamma\delta$ T-cells in mice must recognize a self-component that selects these cells in the fetal thymus⁷ and regulates their function in the periphery; here the subset would be expressed in response to a large variety of pathogens or non-infectious agents, as previously speculated^{13,14}. Further study should shed light on the role of these epithelia-associated $\gamma\delta$ T-cell subsets in the immune system. □

Rapid neutrophil adhesion to activated endothelium mediated by GMP-140

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GRANULE membrane protein-140 (GMP-140), a membrane glycoprotein of platelet¹⁻⁵ and endothelial cell⁶⁻⁸ secretory granules, is rapidly redistributed to the plasma membrane during cellular activation and degranulation^{1,2,6,7}. Also known as PADGEM protein⁴, GMP-140 (ref. 9) is structurally related to two molecules involved in leukocyte adhesion to vascular endothelium: ELAM-1, a cytokine-inducible endothelial cell receptor for neutrophils¹⁰, and the MEL-14 lymphocyte homing receptor^{11,12}. These three proteins define a new gene family, termed selectins, each of which contains an N-terminal lectin domain, followed by an epidermal growth factor-like module, a variable number of repeating units related to those in complement-binding proteins, a transmembrane domain, and a short cytoplasmic tail. Here we demonstrate that GMP-140 can mediate leukocyte adhesion, thus establishing a functional similarity with the other selectins. Human neutrophils and promyelocytic HL-60 cells bind specifically to COS cells transfected with GMP-140 complementary DNA and to microtitre wells coated with purified GMP-140. Cell binding does not require active neutrophil metabolism but is dependent on extracellular Ca²⁺. Within minutes after stimulation with phorbol esters or histamine, human endothelial cells become adhesive for neutrophils; this interaction is inhibited by antibodies to GMP-140. Thus, GMP-140 expressed by activated endothelium might promote rapid neutrophil targeting to sites of acute inflammation.

COS cells transfected with a cDNA encoding GMP-140 support adhesion (rosette formation) of isolated human neutrophils and promyelocytic HL-60 cells: similar adhesion has been observed with ELAM-1-transfected COS cells¹⁰. Immunoperoxidase analysis indicates that the anti-GMP-140 monoclonal antibodies G1, G2, S12 (refs 1, 5) and W40 (ref. 5) identify GMP-140-transfected COS cells, but not COS cells transfected with ELAM-1 or ICAM-1 cDNAs. Figure 1 shows that antibody G1 blocks adhesion of HL-60 cells to GMP-140-transfected COS cells, but not to ELAM-1-transfected COS cells. Conversely, anti-ELAM-1 antibody H18/7 (ref. 13) inhibits binding of HL-60 cells to COS cells expressing ELAM-1, but not to COS cells expressing GMP-140; there is a comparable specific inhibition of neutrophil adhesion. Leukocyte adhesion to GMP-140-transfected COS cells is also inhibited by G2, but not by S12 or W40, suggesting that these two antibodies recognize epitopes not essential for leukocyte binding.

Figure 2 shows that human neutrophils and HL-60 cells bind to microtitre wells coated with purified platelet GMP-140, but not to wells coated with control proteins. This indicates that GMP-140 is sufficient to promote leukocyte adhesion. Neutrophil binding is blocked by preincubation of GMP-140-

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coated wells with the anti-GMP-140 monoclonal antibodies G1, G2, and G3 (Fig. 3a), and with polyclonal antibodies to GMP-140 (not shown). Half-maximal inhibition of cell binding is observed at antibody concentrations of 5–10 $\mu\text{g ml}^{-1}$. Adhesion is not blocked by control antibodies or by anti-GMP-140 antibodies S12 and W40. Fluid-phase GMP-140, but not other proteins, inhibit cell binding to immobilized GMP-140 (Fig. 3b). This suggests that there are a saturable number of cell-surface binding sites that can be occupied by either fluid-phase or immobilized GMP-140.

Paraformaldehyde-fixed neutrophils and HL-60 cells bind to GMP-140, indicating that the cells do not need to be metabolically active to express a functional surface ligand for GMP-140 (Fig. 2). This contrasts with the active cellular metabolism necessary to support adhesive activity of leukocyte integrins¹⁴. Furthermore, cell binding to GMP-140 is not inhibited by IB4 (ref. 15), a monoclonal antibody against the β -chain of leukocyte integrins which blocks their known adhesive functions (Fig. 3a). This indicates that these molecules are probably not ligands for GMP-140.

As GMP-140 contains an N-terminal domain homologous to a family of Ca^{2+} -dependent lectins¹⁶, we tested the effects of divalent cations on neutrophil binding to GMP-140. Varying amounts of EGTA were used to titrate the free Ca^{2+} concentration from 10^{-9} M to 10^{-3} M, in the presence or absence of 10^{-3} M free Mg^{2+} (Fig. 4). In the absence of either divalent cation, binding of neutrophils to GMP-140 is completely prevented. Addition of increasing amounts of Ca^{2+} supports progressively more adhesion, with maximal neutrophil binding at 10^{-3} M Ca^{2+} . Higher Ca^{2+} levels did not support more adhesion. Addition of 10^{-3} M Mg^{2+} also supported neutrophil binding to GMP-140, but only when Ca^{2+} was present at levels of 10^{-6} M or greater. These results indicate that there are at least two divalent-

cation binding sites that must be occupied to support optimal neutrophil adhesion to GMP-140, a high-affinity site specific for Ca^{2+} and a lower affinity site recognized by either Ca^{2+} or Mg^{2+} . The MEL-14 lymphocyte homing receptor, another selectin, also requires Ca^{2+} to mediate cell interactions¹⁷. But, unlike binding mediated by GMP-140, low levels of Ca^{2+} will not allow Mg^{2+} to support adhesion by the MEL-14 receptor¹⁷.

In vivo, neutrophils bind to endothelium within minutes after application of certain inflammatory stimuli, an event which has been attributed to an alteration in the endothelial surface¹⁸. *In vitro*, certain agonists such as thrombin induce endothelium to become adhesive for neutrophils within minutes^{19,20}. To investigate the potential role of GMP-140 in these adhesive interactions, we measured binding of neutrophils to human endothelial monolayers stimulated with phorbol esters or histamine, both of which cause rapid expression of GMP-140 on the endothelial surface^{6,7}. Treatment of endothelium with 10^{-9} M to 10^{-6} M 4 β -phorbol 12-myristate 13-acetate (PMA), followed by washing, causes enhanced neutrophil adhesion that is measurable

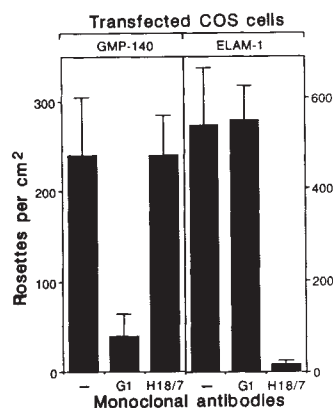


FIG. 1 HL-60 cell adhesion to COS cells transfected with cDNAs encoding GMP-140 or ELAM-1. Anti-GMP-140 antibody G1 inhibits adhesion to GMP-140-transfected COS cells but not to ELAM-1-transfected COS cells. Conversely, anti-ELAM-1 antibody H18/7 inhibits adhesion to ELAM-1-transfected COS cells, but not to GMP-140-transfected COS cells.

METHODS. A full-length cDNA for GMP-140 (E4 in ref. 9) was isolated from PBL-20 by *SalI* digestion. After blunt-ending and addition of *Bst*X1 linkers, it was ligated into the CDM8 expression vector. COS cells were transfected with either GMP-140 or ELAM-1 cDNA in CDM8 using DEAE-dextran, then trypsinized and transferred to experimental wells 48 h before the adhesion assay¹⁰. After incubation of COS cell monolayers for 45 min in medium with or without antibody, HL-60 cells were added for 30 min. After washing, adhesion to the monolayer was measured by counting the number of HL-60 cell rosettes (four or more HL-60 cells per COS cell) in five low-power microscopic fields on each of three coverslips. G1 was used as culture supernatant (neat); similar inhibition of adhesion was seen in other experiments using purified IgG (see legend to Fig. 3). H18/7, fractionated from ascites, was used at $10 \mu\text{g ml}^{-1}$. Control antibody either as culture supernatants or fractionated ascites had no effect on adhesion to either type of transfected COS cell. Error bars, standard deviation.

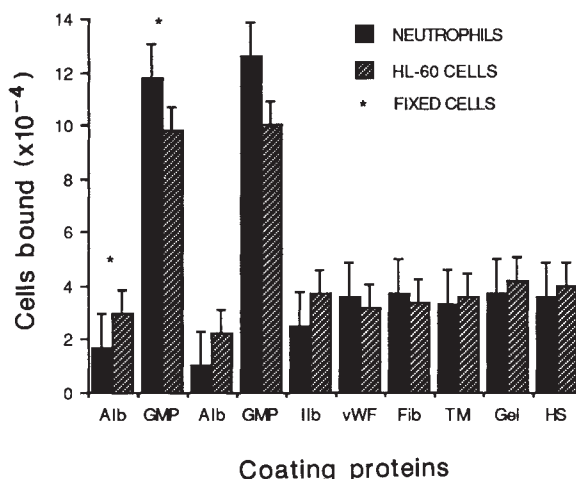


FIG. 2 Neutrophils and HL-60 cells bind to wells coated with purified GMP-140 but not to wells coated with other proteins.

METHODS. Human neutrophils were isolated from heparinized whole blood by density gradient centrifugation (Mono-Poly resolving media, Flow). Neutrophil suspensions were >98% pure and >95% viable by trypan blue exclusion. Human HL-60 cells were maintained in RPMI-1640 with 10% fetal bovine serum. For adhesion assays, neutrophils or HL-60 cells were suspended at a concentration of 2×10^6 cells ml^{-1} in Hanks' balanced salt solution containing 1.26 mM Ca^{2+} and 0.81 mM Mg^{2+} (HBSS, GIBCO) with 5 mg ml^{-1} human serum albumin (HBSS/HSA). In certain assays cells were fixed for 1 h at 4°C with 1% paraformaldehyde in HBSS buffered at pH 7.4 with 20 mM HEPES, then washed 3 times with HBSS/HEPES. Adhesion assays were in triplicate in 96-well microtitre plates (Corning) incubated at 4°C overnight with 50 μl of various protein solutions. GMP-140 was purified from human platelet lysates by immunoaffinity chromatography on antibody S12-Sepharose^{1,5} and ion-exchange chromatography on a Mono-Q column (FPLC, Pharmacia). GMP-140 (GMP) was plated at $5 \mu\text{g ml}^{-1}$, and the control proteins human serum albumin (Alb), platelet glycoprotein IIb/IIIa (Ilb), von Willebrand factor (vWF), fibrinogen (Fib), thrombomodulin (TM), gelatin (Gel) or human serum (HS) were placed at $50 \mu\text{g ml}^{-1}$. All wells were blocked for 2 h at 22°C with 300 μl HBSS containing 10 mg ml^{-1} HSA, then washed 3 times with HBSS containing 0.1% Tween-20 and once with HBSS. Cells (2×10^5 per well) were added to the wells and incubated at 22°C for 20 min. The wells were then filled with HBSS/HSA, sealed with acetate tape (Dynatech) and centrifuged inverted at 150g for 5 min. After discarding nonadherent cells and supernates, the contents of each well were solubilized with 200 μl 0.5% hexadecyltrimethylammonium bromide (Sigma) in 50 mM potassium phosphate, pH 6.0, and assayed for myeloperoxidase activity²⁶. The number of cells bound was derived from a standard curve of myeloperoxidase activity vs numbers of cells. Under all assay conditions, the cells released less than 5% of total myeloperoxidase and lactate dehydrogenase. Results are presented as numbers of cells bound. Error bars, standard deviation.

within 10 min and reaches a maximum at 30 min. This adhesion is blocked by pretreatment of endothelial cells with the protein kinase C inhibitor, staurosporine. Neutrophil binding to PMA-stimulated endothelium is inhibited by anti-GMP-140 antibody G1 but not by S12 or a control antibody (Fig. 5). Monoclonal antibodies G2 and G3 and polyclonal antibodies against GMP-140 also inhibit adhesion (results not shown). The enhanced neutrophil binding is not blocked by pretreatment of endothelium with actinomycin D, an inhibitor of RNA synthesis, or by cycloheximide or emetine, inhibitors of protein synthesis. ELAM-1 is not likely to mediate the observed neutrophil-endothelial interaction, because it is not present in unstimulated endothelium and requires new protein synthesis for expression^{10,13}. Furthermore, anti-ELAM-1 antibody H18/7 does not block neutrophil adhesion to endothelium stimulated with PMA under the same conditions as those in Fig. 5, although

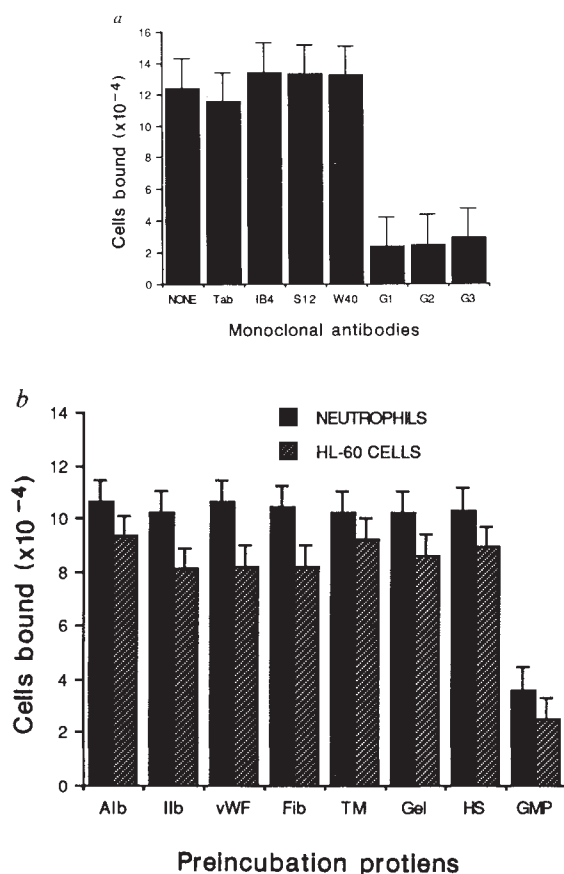


FIG. 3 *a*, Monoclonal antibodies against GMP-140 inhibit neutrophil adhesion to wells coated with GMP-140. *b*, Fluid-phase GMP-140 inhibits adhesion of neutrophils and HL-60 cells to wells coated with GMP-140.

METHODS. Adhesion to GMP-140-coated wells was measured as described in Fig. 2, except that a preincubation step was added. In *a*, before addition of neutrophils, GMP-140-coated wells were incubated for 1 h at 22 °C with 50 μ l purified antibody at final concentrations of 50 μ g ml⁻¹. The antibodies were Tab, directed against platelet glycoprotein IIb (ref. 27); IB4, which recognizes the β -chain of the leukocyte integrins¹⁵; anti-GMP-140 antibody S12 (refs 1, 5) and W40 (ref. 5); and G1, G2, and G3, three new IgG1 κ monoclonal antibodies produced by hybridomas from a mouse immunized with purified human platelet GMP-140. Each anti-GMP-140 antibody identifies a distinct epitope, based on analysis of competitive binding to GMP-140. Antibodies were purified from murine ascites by ammonium sulphate precipitation and quaternary aminoethyl Sephadex (Sigma) chromatography. In *b*, cells were preincubated for 20 min at 22 °C in the presence of the indicated protein (see legend to Fig. 2 for key to abbreviations) at a concentration of 50 μ g ml⁻¹ in HBSS/HSA, then transferred to GMP-140-coated wells for an additional 20-min incubation. Preincubation of cells with the membrane proteins glycoprotein IIb/IIIa, thrombomodulin, or GMP-140 resulted in final concentrations of nonionic detergents of 0.003% to 0.01%; these concentrations of detergent did not affect cell adhesion.

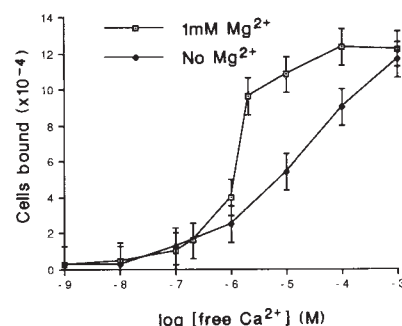


FIG. 4 Divalent-cation dependence of neutrophil adherence to GMP-140. Neutrophils were washed and resuspended in Chelex (Bio-Rad)-treated HBSS/HSA, which contained 47.5 μ M Ca²⁺ and 2.8 μ M Mg²⁺ as determined by atomic absorption spectrophotometry (Varian Techtron, Model 1200). Neutrophil binding to GMP-140-coated wells was performed in Chelex-treated HBSS/HSA at various free Ca²⁺ concentrations in the presence or absence of 1 mM Mg²⁺. The amounts of Ca²⁺, Mg²⁺, and EGTA required to achieve the indicated free divalent cation concentrations were determined using the FREECAL program²⁸. Ultrapure Ca²⁺ and Mg²⁺ (>99.99% purity) were obtained from Garland-Schlesinger.

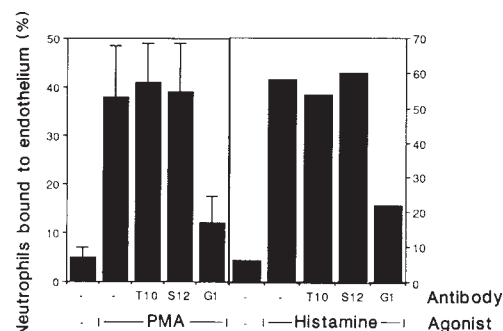


FIG. 5 Anti-GMP-140 antibody G1 inhibits neutrophil adhesion to endothelium activated with PMA or histamine. Left panel, G1 but not S12 or the isotype-matched control antibody T10 (ref. 27), inhibits neutrophil binding to endothelium stimulated with 10⁻⁷ M PMA for 30 min. The bars indicate results (mean and standard deviation) from 4 experiments. Right panel, G1, but not S12 or T10, inhibits neutrophil binding to endothelium stimulated with 10⁻⁴ M histamine for 10 min. The bars indicate values from single points in a representative experiment.

METHODS. Primary postconfluent monolayers of human umbilical vein endothelial cells were cultured in 22-mm cluster dishes¹⁹. The monolayers were washed with HBSS and then incubated in HBSS/HSA with control buffer, PMA, or histamine for the times indicated in an atmosphere of 5% CO₂, 95% air at 37 °C. Dimethylsulphoxide, the solvent for PMA, was included in control buffers when PMA was used as the endothelial cell agonist. When monoclonal antibodies were used, they were added to endothelial cells at a final concentration of 10 μ g ml⁻¹ before addition of the agonist and were present throughout the incubation. After the stimulation period, incubation mixtures were removed from the monolayers, and human neutrophils (0.5 ml of a suspension containing 5.5 $\times 10^6$ cells ml⁻¹ in HBSS/HSA, radiolabelled as described in ref. 19) were incubated with monolayers for 5 min without agitation at 37 °C in 5% CO₂, 95% air. The same antibody used for endothelial preincubation was included in the neutrophil suspensions at 10 μ g ml⁻¹. After the 5-min incubation period, nonadherent neutrophils were removed. The adherent fraction was measured¹⁹ and expressed as a percentage of the total neutrophils added. Inspection of adherent cells by phase contrast microscopy indicated that neutrophils were bound to the surface of endothelial cells. Retraction of endothelial cells leading to exposure of subendothelial matrix was not observed.

it inhibits neutrophil adhesion to endothelium activated by a 4-hour treatment with tumour necrosis factor.

Incubation of endothelium with histamine causes time-dependent neutrophil adhesion which reaches a maximum after 5–10 minutes and then declines. Histamine does not induce cultured smooth muscle cells to become adhesive for neutrophils, nor does it directly stimulate neutrophils to adhere to gelatin, laminin or fibronectin matrices. Antibody G1 inhibits neutrophil binding to histamine-activated endothelium, but S12 and a control antibody do not (Fig. 5). Also, adhesion is blocked by G2 and G3 antibodies and by polyclonal antibodies to GMP-140 (data not shown).

Platelet-activating factor (PAF), a phosphoglyceride, has also been implicated in rapid endothelial cell-dependent neutrophil adhesion^{19–21}. PMA does not induce endothelial cell synthesis of PAF (ref. 22, and unpublished data), indicating that this lipid is not involved in PMA-induced adhesiveness of endothelium for neutrophils. But physiological agonists such as histamine and thrombin cause rapid and transient expression of both GMP-140 (refs 6, 7) and PAF^{21,23} on the endothelial cell surface. At sites of acute inflammation where these agonists are present, coordinate endothelial expression of GMP-140 and PAF may mediate rapid, regulated neutrophil adherence and activation within minutes after the endothelium is stimulated. In contrast, inflammatory mediators such as tumour necrosis factor or interleukin-1 induce endothelial synthesis of ELAM-1, with maximal expression after several hours^{10,13}. Therefore, transport of ELAM-1 to the endothelial cell surface may mediate more gradual and sustained neutrophil adherence.

GMP-140 is also expressed on the surface of stimulated platelets^{1–4}, where it mediates platelet-leukocyte adhesive interactions^{24,25}. By promoting rapid binding of leukocytes to both activated platelets and endothelium, GMP-140 may provide an important link between the haemostatic and inflammatory responses to tissue injury. □

Molecular components of the B-cell antigen receptor complex of the IgM class

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THE antigen receptors on mature B lymphocytes are membrane-bound immunoglobulins of the IgM and IgD classes^{1,2} whose cross-linking by polyvalent antigens results in B-cell proliferation and differentiation³. How these membrane-bound immunoglobulin chains, which lack a cytoplasmic tail, generate a cell activation signal is not at present known. We now show that the IgM molecule is non-covalently associated in the membrane of B cells with two proteins of relative molecular mass 34,000 (M_r 34 K; IgM- α) and 39 K (Ig- β) which form a disulphide-linked heterodimer. Surface expression of IgM seems to require the formation of an appropriate complex between IgM and the heterodimer. A transfection experiment indicates that IgM- α is the product of *mb-1*, a B-cell-specific gene⁴ encoding a transmembrane protein with sequence homology⁵ to proteins of the T-cell antigen receptor-CD3 complex^{6–8}.

J558L myeloma cells, transfected with expression vectors coding for the membrane-bound form of the μ -chain (μ_m), do not show expression of surface IgM (sIgM) although complete IgM molecules are assembled intracellularly^{9,10}. By using a cell sorter we have previously isolated the sIgM⁺ myeloma variant J558L μ_m 3, showing that this variant differs from the μ_m transfectant (J558L μ_m) in containing transcripts of the *mb-1* gene and a glycoprotein of M_r 34K (B34, now called IgM- α) which is co-purified with membrane-bound IgM molecules¹¹.

To elucidate the relationship between the expression of *mb-1*, IgM- α and sIgM, we constructed the vectors pSVmb-1m and pSVmb-1h (Fig. 1e), which express the mouse and human *mb-1* genes, respectively. We introduced these vectors into the myeloma line J558L μ_m which expresses membrane-bound IgM molecules without bringing them onto the cell surface (Fig. 1a). We analysed the transfectants (J558L μ_m /mb-1) for the presence of *mb-1* transcripts (data not shown) and for sIgM expression. AU *mb-1*-expressing transfectants were >90% sIgM⁺. Mouse *mb-1* transfectants (J558L μ_m /mb-1m) expressed sIgM as strongly as the myeloma variant line J558L μ_m 3 (Fig. 1b, d), whereas sIgM expression of human *mb-1* transfectants (J558L μ_m /mb-1h) was 5–10 times lower (Fig. 1c).

We then tested the J558L μ_m line and the J558L μ_m /mb-1m transfectant for the presence of IgM-associated proteins. We analysed biosynthetically labelled and affinity-purified IgM antigen receptors of both cells by two-dimensional nonreducing-reducing SDS-PAGE (Fig. 2). In this analysis, monomeric proteins are found on the diagonal of the gel, whereas subunits of disulphide-bound proteins appear below the diagonal. The purified receptors of J558L μ_m /mb-1m contained several disulphide-linked proteins (Fig. 2a). These were μ_m and λ -chains from H₂L₂ and HL complexes as well as the 34K protein IgM- α , which we have also previously found in the sIgM⁺ variant line J558L μ_m 3 (ref. 11). The IgM- α protein was only present in J558L μ_m /mb-1m and not in the J558L μ_m recipient line (Fig. 2b).

We have previously interpreted the disulphide-linked IgM- α proteins as a homodimer¹¹. But a close inspection of the two-dimensional gel in Fig. 2a revealed a faint diffuse protein band of $M_r \sim 39$ K above the IgM- α protein. This protein band was better seen after silver staining of a two-dimensional gel containing large amounts of affinity-purified IgM antigen receptor of the J558L μ_m 3 line (Fig. 3a). Indeed, the 39K protein (called Ig- β) and the 34K IgM- α protein were stained with similar intensity. Because both proteins lay above each other and below

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the diagonal of the gel, they seemed to form a heterodimer. To test whether the heterodimer was expressed on the cell surface, we surface-iodinated J558L μ_m 3 cells and analysed their antigen receptor on a two-dimensional gel (Fig. 3b). Both proteins of the heterodimer were labelled, indicating that they are expressed together with the IgM molecule on the cell surface.

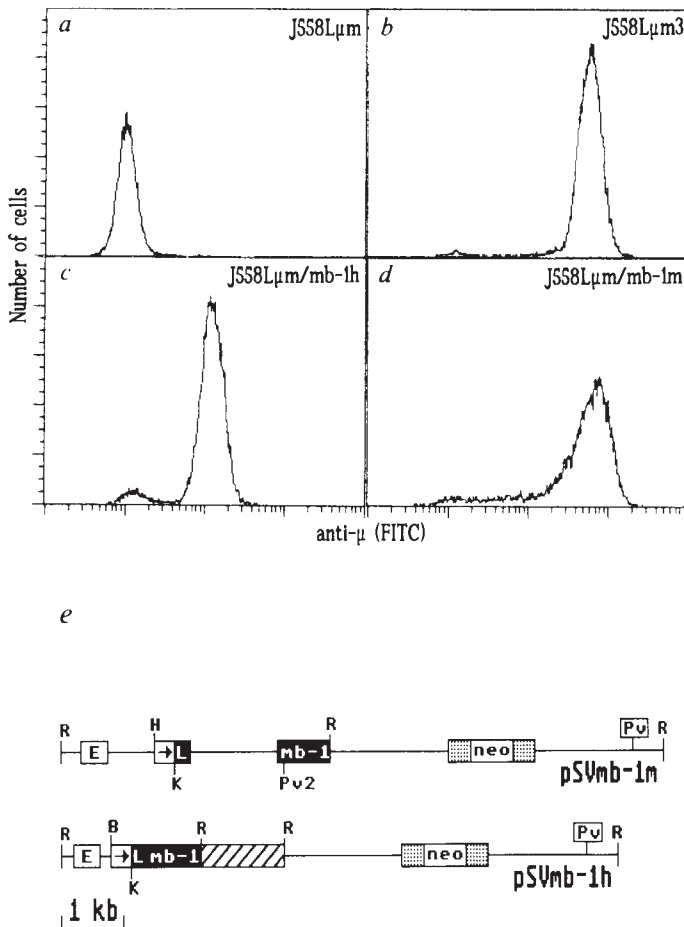


FIG. 1 Surface IgM expression of μ_m -transfected myeloma cells requires expression of the *mb-1* gene. Flow cytometric analysis of surface IgM expression of the μ_m transfectant J558L μ_m (a), the sIgM⁺ variant J558L μ_m 3 (b), the human *mb-1* transfectant J558L μ_m /mb-1h (c) or the mouse *mb-1* transfectant J558L μ_m /mb-1m (d). The *neo* vectors used for transfection are shown in (e). Restriction sites: B, *Bam*HI; H, *Hind*III; K, *Kpn*I; Pv, *Pvu*I; Pv2, *Pvu*II; R, *Eco*R1.

METHODS. Cells were stained with fluorescein-labelled goat anti-mouse IgM antiserum (Southern Biotechnology, Birmingham, Alabama). Histograms were generated by the analysis of 10,000 cells. For *mb-1* vector construction, the genomic *Eco*R1 fragments and complementary DNA of mouse *mb-1* were cloned using specific oligonucleotide probes made according to the sequence of ref. 4. Full-length human *mb-1* cDNA was isolated from a cDNA library of Raji B cells with a mouse *mb-1* probe. The mouse vector pSVmb-1m contained a 1.8-kilobase (kb) genomic *Kpn*I–*Pvu*2 fragment including leader, intron and second exon sequences, and a 0.7-kb *Pvu*2–*Eco*R1 cDNA fragment of the *mb-1* gene. Immediately 5' to the *mb-1* sequence, a 1.5-kb *Eco*R1–*Hind*III fragment of the mouse IgH enhancer (E) and a 350-base pair (bp) fragment from a *VH* promoter (indicated by an arrow) were inserted. The correctly assembled fragment was cloned into the *Eco*R1 site of the pSV2neo vector²⁰. The human vector pSVmb-1h contained the human *mb-1* cDNA on a *Kpn*I–*Eco*R1 fragment. Immediately 5' was the IgH enhancer and *VH* promoter (see above). The 3' end of the *mb-1* sequence was fused to a fragment carrying a splice and poly(A) site from the nontranslated 3' region of the simian virus 40 (SV40) genome (stripped box). The assembled fragments were cloned into the *Eco*R1 site of the pSV2neo vector. The *mb-1* vectors were transfected by electroporation as described previously¹¹.

We next investigated whether the IgM antigen receptor of B lymphoma cells has the same composition as that of the sIgM⁺ myelomas studied so far. We co-transfected the B lymphoma cells K46 (ref. 12) with the vectors pIXE (ref. 13) and pSV μ_m (ref. 14) encoding a λ_1 and a μ_m chain, respectively. In the K46 $\lambda\mu_m$ transfectants, IgM molecules were brought immediately onto the cell surface (data not shown). After purification, we analysed the IgM antigen receptor of K46 $\lambda\mu_m$ on two-dimensional gels as described above. The two proteins of the heterodimer were detected by either silver staining (Fig. 3c) or surface iodination (Fig. 3d).

We demonstrated the specificity of binding between the μ_m chain and the heterodimer by introducing the variant vector pSV μ_{Tm} (Fig. 3f) into K46 λ cells. This vector expresses a chimaeric μ -chain in which the μ_m transmembrane part is replaced by the transmembrane region and the cytoplasmic tail of the major histocompatibility (MHC) class-I (H-2K^k) molecule¹⁵. The chimaeric μ_{Tm} chain comes to the cell surface of myeloma transfectants independently of *mb-1* expression (data not shown). The surface labelled IgM molecules of K46 $\lambda\mu_{Tm}$ cells were not associated with the heterodimer (Fig. 3e). This result demonstrates that the μ_m chains bind the heterodimer only if they contain their own transmembrane part.

We have shown here that IgM molecules are associated in the B-cell membrane with at least two other proteins, IgM- α and Ig- β , forming a disulphide-linked heterodimer. Therefore the IgM antigen receptor on B cells seems to be a complex of different transmembrane proteins (IgM- α , Ig- β , μ_2) and, as such, is similar to the antigen receptor on T cells^{6–8} and the Fc receptor for the IgE on mast cells¹⁶. The surface expression of these receptors requires the complete assembly from their respective subunits^{16,17}. This is seen for the IgM antigen receptor in μ_m -transfected myeloma cells, where the absence of one component results in a block of surface expression. This block is released on transfection with vectors expressing *mb-1*. The *mb-1* gene therefore seems to encode one of the IgM-associated proteins. In other studies we show that the IgD antigen receptor also contains a heterodimer that seems to have the same β -component but a different α -component from that of the IgM antigen receptor¹⁸. In sIgD-expressing myeloma cells, the *mb-1*

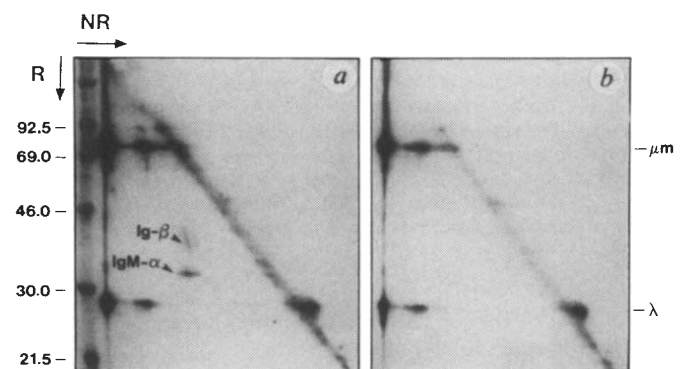


FIG. 2 Two-dimensional (nonreducing (NR)–reducing (R)) gel analysis of [³⁵S]methionine-labelled IgM antigen receptors from the myeloma transfectants J558L μ_m /mb-1m (a) and J558L μ_m (b). The arrows indicate the position of IgM- α and Ig- β proteins.

METHODS. Cells were biosynthetically labelled with [³⁵S]methionine and then lysed with 1% digitonin. The IgM molecules expressed in J558L μ_m lines have a nitrophenol (NP) binding specificity and were purified from the digitonin lysates by affinity chromatography over NP-Sepharose columns. Proteins were first size-separated on a 10% SDS-polyacrylamide tube gel without prior reduction. After reduction, gels were placed on 10% SDS-polyacrylamide slab gels and run in the second dimension. For detailed description of cell labelling, lysis and receptor purification, see ref. 11.

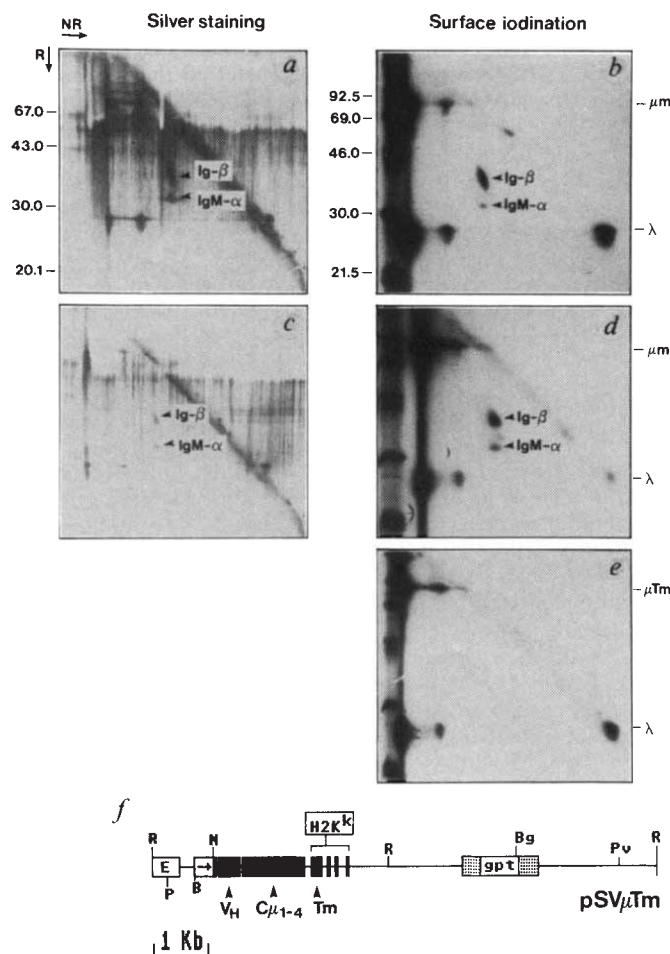


FIG. 3 Two-dimensional (nonreducing-reducing) gel analysis of purified IgM antigen receptor of the myeloma variant J558L μ _m3 (a, b) and the B-cell lymphoma transfectants K46 λ μ _m (c, d) and K46 λ μ T_m (e). Receptor proteins are identified either by silver staining (a and c) or by cell surface iodination (b, d and e). M_r ($\times 10^{-3}$) indicated on left of panel. Position of the IgM- α and IgM- β protein is indicated by arrows. Restriction map of chimaeric μ -vector pSV μ T_m (f). Restriction sites: B, BamHI; H, HindIII; K, KphI; Pv2, Pvu2; Pv, PvuI; R, EcoRI.

METHODS. For cell surface iodination, 5×10^7 cells were labelled with 1 mCi Na¹²⁵I using the lactoperoxidase-glucose oxidase method²¹ and lysed with 1% digitonin. For silver-stained gels, 10^9 cells were disrupted by sonification in sucrose buffer (10 mM Tris buffer, pH 7.4, 8% sucrose, and protease inhibitors). Cellular debris was pelleted by centrifugation at 40,000 r.p.m. and lysed in 1% digitonin. The IgM molecules were purified from the lysates over NP-Sepharose columns as previously described¹¹. Nonreduced samples were first run on 10% tube gels and, after reduction, on 12% slab gels. Silver staining was performed according to ref. 22.

gene is not expressed, and *mb-1* therefore presumably encodes the IgM-specific IgM- α component.

A detailed molecular analysis of the IgM-associated proteins could help in the understanding of signal transduction from the IgM antigen receptor. As judged from the *mb-1* sequence, the IgM- α protein has a 60-amino-acid cytoplasmic tail carrying tyrosines in a sequence motif conserved between B- and T-cell receptor molecules⁵. This motif could be part of the binding site for the G protein, which is postulated to participate in signal transduction¹⁹. □

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Activation of cell growth by binding of Friend spleen focus-forming virus gp55 glycoprotein to the erythropoietin receptor

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FRIEND spleen focus-forming virus (SFFV) is a defective murine C-type retrovirus which causes a multi-stage erythroleukaemia in mice and erythroblastosis in bone marrow cultures¹⁻⁵. The SFFV *env* gene encodes a membrane glycoprotein, gp55, which is located on the cell surface and in the rough endoplasmic reticulum⁶⁻⁸ and is essential both for the induction of leukaemia *in vivo*⁹⁻¹³ and erythroblast proliferation *in vitro*^{1,4}. The mechanism by which gp55 causes increased erythroblastosis and ultimately leukaemia is unknown, but a reasonable suggestion is that gp55 can mimic the action of erythropoietin by binding to its receptor (Epo-R), thereby triggering prolonged proliferation of erythroid cells. To test this possibility, we have co-expressed gp55 and the murine Epo-R in a fibroblast cell line. We show here that in such cells, the SFFV glycoprotein binds directly to Epo-R. Furthermore, when an interleukin-3 (IL-3)-dependent lymphoid cell line was co-infected by SFFV and a virus that carries the Epo-R gene, it could grow without IL-3. We suggest that through direct binding to Epo-R, gp55 can stimulate the receptor and by-pass the normal requirement for Epo, causing prolonged proliferation of infected erythroid cells. This could be the first step of leukaemogenesis induced by Friend virus.

To express Epo-R in fibroblast cells, we constructed plasmid pSF-ER (Fig. 1) and transfected it into a mixture of psi-cre and psi-crip fibroblast packaging lines¹⁴. After two weeks of incubation, the cells made a substantial amount of Epo-R (Fig. 2a, lane 8), expressing an average of 2×10^5 surface Epo-R molecules per cell, and produced infectious virus (termed SF-ER; data not shown). The plasmid pL2-6k (Fig. 1; ref. 13) containing the polycythemic Friend SFFV proviral DNA was similarly transfected into the mixed packaging cells and a high

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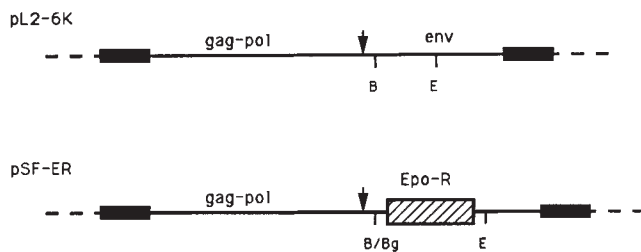


FIG. 1 The plasmid constructs used to express SFFV gp55 or Epo-R in fibroblast cells. The construction of plasmid pL2-6k has been described¹³. For the construction of pSF-ER, the vector pSFF (ref. 23) was cut with enzymes *Bam*HI and *Eco*RI and ligated with the *Bgl*II (compatible with *Bam*HI site)-*Eco*RI fragment of the Epo-R complementary DNA clone 190 (ref. 15). Plasmids were transfected into the fibroblast packaging mixture and cultures were maintained for two weeks to allow the virus to spread through a 'ping-pong' process between packaging cells of different host range²³. Arrows indicate the splice acceptor site in the SFFV RNA genome.

level of gp55 expression was obtained (Fig. 2a, lane 2). The two cultures were mixed together and co-cultivated for a month to allow the two viruses to spread, and individual cell clones were isolated.

One of the clones, clone 2, synthesizes both gp55 and Epo-R (Fig. 2a, lane 1 and 7, respectively). Immunoprecipitation with either anti-glycoprotein or anti-Epo-R antiserum shows that antibody against one protein co-precipitates the other (Fig. 2a, lane 1 and 7), indicating that gp55 and Epo-R might be tightly associated in these cells. To confirm this finding, we first immunoprecipitated the proteins with anti-glycoprotein, then dissociated the precipitated proteins and subjected the soluble protein to a second immunoprecipitation using anti-Epo-R. Only Epo-R protein from clone 2 could be detected in the final precipitate (Fig. 2a, lane 6). A similar observation was made for several other independent fibroblast clones that expressed both gp55 and Epo-R (data not shown). The protein was not present in the final precipitate from cells that made Epo-R alone, (Fig. 2a, lane 5), presumably because it remained in the supernatant during the first immunoprecipitation by anti-

glycoprotein. As further evidence for the association between the two proteins, we first immunoprecipitated the proteins with anti-Epo-R and then with anti-glycoprotein. From clone-2 cells expressing gp70 and gp55, only gp55 is recovered in the final precipitate (Fig. 2b, lane 1). From cells expressing gp55 but not Epo-R, no gp55 is evident in the final precipitate (lane 2). These results show that gp55 interacts strongly with Epo-R.

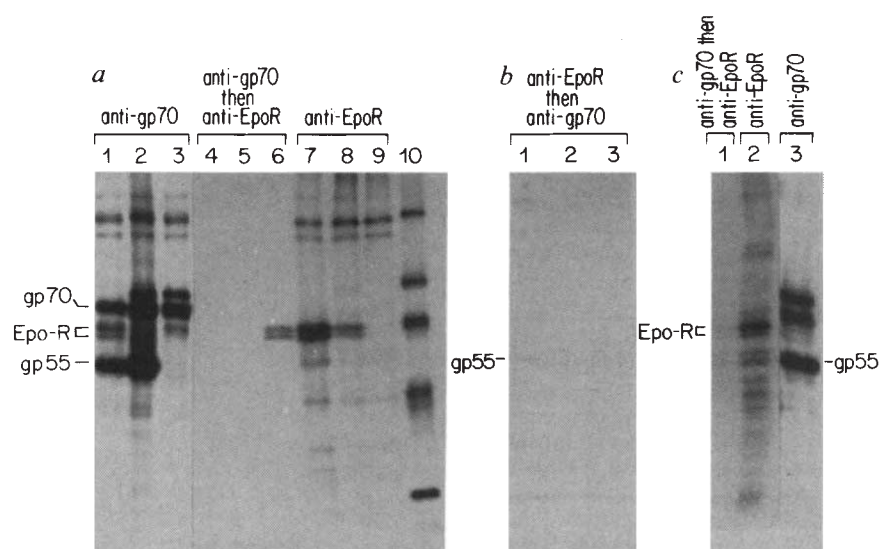
To investigate whether the same interaction occurs in Friend erythroleukaemia (MEL) cells, we extracted their proteins and immunoprecipitated them. When individual antiserum was used, there was little cross-precipitation (Fig. 2c, lanes 2 and 3), probably because the concentration of Epo-R is low in these cells¹⁵. Double-immunoprecipitation using first anti-glycoprotein and then anti-Epo-R, however, revealed some Epo-R in the final precipitate (Fig. 2c, lane 1), indicating that in these cells there is gp55 bound to Epo-R.

We demonstrated that the binding of gp55 to Epo-R is growth-promoting by co-infecting an IL-3-dependent lymphoid cell line, Ba/F3 (ref. 16) with SFFV and SF-ER. Ba/F3 has an absolute dependence on IL-3 for growth¹⁶, but is released from this dependence after co-infection with SFFV and SF-ER, but not by a single infection with either virus alone (Table 1). Ba/F3 can also be converted to Epo-dependence either after transfection with a plasmid containing the Epo-R gene (A. D. D'A., unpublished observation) or by a single infection with SF-ER (Table 1). When the Ba/F3 cells are cloned after co-infection, most of the individual clones are growth factor-independent and express both gp55 and Epo-R, but ~1 in 6 require Epo for growth and express only Epo-R and not gp55 (data not shown). It is evident that both gp55 and Epo-R are required to promote factor-independent growth of these cells.

Previous experiments have indicated that SFFV gp55 plays a crucial part in the initiation of the leukaemogenic process⁹⁻¹³, and that infection by SFFV causes Epo-independent erythroid burst formation *in vitro*⁴ and Epo-independent erythropoiesis *in vivo*¹⁷. Our results indicate that gp55 binds directly to Epo-R and that the mitogenic effect of this interaction can promote the proliferation of even the non-erythroid Ba/F3 cells. Binding of gp55 to Epo-R might then be the key event for triggering the prolonged proliferation of erythroblasts, leading to the activation of additional cellular oncogenes, such as Spi-1 (ref. 18), and eventually to the development of leukaemia. Because all

FIG. 2 SDS-PAGE of [³⁵S]methionine-labelled proteins after immunoprecipitation IP. a, Lanes 1-3, single IPs by a goat anti-Rauscher MuLV gp70 antiserum which cross-reacts with SFFV gp55 (ref. 7) and also with the amphotropic gp70 (seen in lane 2 and lane 3 only); lanes 4-6, double IPs first by anti-gp70 antiserum and then by an antiserum against the N-terminal (APSPSLDPKFKESKC) peptide of the Epo-R protein¹⁵; lanes 7-9, single IPs by anti-Epo-R antiserum. Labelled proteins were extracted from 5×10^5 cells containing pL2-6k (lane 2, 4 and 9), pSF-ER (lane 3, 5 and 8), or proviruses of both constructs (clone 2, lanes 1, 6 and 7). b, Double IPs first by anti-Epo antiserum and then by anti-gp70 antiserum. Lane 1, clone 2; lane 2, cells with pSF-ER; lane 3, cells with pL2-6k. c, Labelled proteins extracted from Friend MEL cells (5×10^6 cells for double IP, 2.5×10^6 cells for anti-Epo-R IP and 5×10^5 cells for anti-gp70 IP). Lane 1, double IP first by anti-gp70 antiserum and then by anti-Epo-R antiserum. Lane 2, single IP by anti-Epo-R antiserum. Lane 3, single IP by anti-gp70 antiserum.

METHODS. The general method of immunoprecipitation (single IP) was described previously⁷. For double IPs, the [³⁵S]methionine-labelled cells were lysed with IP buffer (20mM Tris, pH 7.4, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, 0.5 M NaCl, and 1 mM EDTA) and the protein extracts diluted according to the relative signal intensities from a single IP. After the first IP (the procedure was the same as in single



IPs), the IP pellets were resuspended in 0.05 ml loading buffer (60 mM Tris, pH 6.8, 2% SDS, 10% glycerol, and 1% 2-mercaptoethanol) and heated at 100°C for 10 min. After a brief microcentrifugation, the supernatants were diluted 1:20 with IP buffer and subjected to a second IP using a different antiserum.

TABLE 1 Growth of Ba/F3 cells after infection*

Ba/F3 cells infected by	Medium supplemented with		
	IL-3	Epo	None
None	+	—	—
SFFV	+	—	—
SF-ER	+	+	—
SFFV + SF-ER	+	+	+

* About 5×10^5 Ba/F3 cells were infected with SFFV, SF-ER, or both. After infection, the cells were kept for 24 h in IL-3 (10% WEHI-3 supernatant) before switching to either erythropoietin (Epo; 0.5 units per ml) or regular medium without any added growth factors. Positives (+) represent cultures (of 5×10^5 cells) with rapidly growing cells that became established within one week of infection. Negatives (—) represent cultures with no viable cells left within 3–4 days after infection and no cell growth even after up to one month of incubation.

Friend erythroleukaemia cell lines tested continue to express gp55 when maintained in culture while expression of other virus-encoded proteins may cease¹⁹, interaction between gp55 and Epo-R may still be necessary to maintain the malignant status of late-stage leukaemic cells; our results indicate that this protein interaction does persist in these cells. It is the intracellular form of gp55, and not the larger form localized on the cell surface, that is bound to Epo-R. Therefore, the interaction between the two proteins probably occurs in the endoplasmic reticulum. Because the plasma membrane form constitutes only ~5% of the total SFFV glycoprotein⁶, its possible binding to Epo-R would be difficult to detect.

The ability of gp55 to stimulate cell proliferation by binding to the erythropoietin receptor represents a novel mechanism by which viruses can override normal growth-regulating functions. It will be of interest to see whether other viruses use similar mechanisms to cause proliferation of specific cell types. For instance, the envelope glycoproteins encoded by several oncogenic mink cell focus-forming viruses (MCFs) are structurally related to SFFV gp55 (refs 20, 21). Because the amino-acid sequence of Epo-R is similar to that of the β -subunit of the IL-2 receptor²², it is possible that the MCF envelope glycoprotein interacts with the IL-2 receptor or another member of this group of growth factor receptors, thereby causing MCF-induced T-cell leukaemia. □

Ion channels in the nuclear envelope

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CELL nuclei are capable of partitioning a wide variety of molecules from the cytosol, including macromolecules such as proteins^{1–11} and RNA^{12–14}, and smaller peptides^{9,14–16}, amino acids¹⁷, sugars^{18,19} and Na⁺ and K⁺ ions^{20,21}, all of which can be accumulated in or excluded from the nuclear domain. There are two mechanisms behind this compartmentalization: selective retention of freely diffusible molecules, and selective entry through the nuclear envelope. It is generally accepted that the nuclear envelope restricts only the larger molecules^{22–24}. Here we apply the patch-clamp technique to isolated murine pronuclei²⁵ and show that the nuclear envelope contains K⁺-selective channels which have multiple conductance states, the maximal conductance being 200 pS. These channels, which contribute to the nuclear membrane potential²⁶, may be important in balancing the charge carried by the movement of macromolecules in and out of the nucleus.

Nuclear envelopes contain pores for the selection of macromolecules, but these pores are considered to be too large to act as barriers against ions^{22–24}. Nevertheless, electrical potential across nuclear envelopes and segregation of ions in the nucleoplasm have been reported^{21,26,27}, indicating that there could be ion-selective channels in the nuclear membrane. We have repeated the nuclear resting-potential measurements *in situ* in murine zygotes. Using an enucleation procedure²⁵ (Fig. 1), we compare these results with nuclear potentials and patch-clamp recordings in cell-free nuclei.

The average potential between the nucleoplasm and the cytoplasm, from the protocol shown in Fig. 2a, is -10.75 ± 1.25 mV ($n=4$). After placing the zygote in a solution of 120 mM K⁺/0 mM Na⁺ (b) to null its resting voltage, the nuclear potential becomes -9.87 ± 0.85 mV ($n=4$). A cell-free nucleus in the same solution (c) has a potential of -9.5 ± 2.08 mV ($n=4$), and in a 60 mM K⁺/60 mM Na⁺ solution (d) the potential is -23.5 ± 4.2 mV ($n=4$). The hyperpolarization that takes place following this change in the composition of the external solution indicates that a K⁺ selective mechanism could be operating in the nuclear envelope.

To investigate the possibility that this mechanism involves ion-selective channels, we formed nuclear-attached patches on extracted pronuclei. Figure 3a shows patch currents at pipette voltages (V_p) equal to +20, -20, +30 and -30 mV. Examples of the single-channel records at $V_p = -10$ mV are shown in d on a faster timescale. To obtain the absolute voltage, we used $V = (V_{rest} - V_p)$, where V_{rest} is the nuclear potential from Fig. 2c. From the current-voltage ($i(V)$) curves in Fig. 3c, which are obtained by plotting the currents at the peaks of the amplitude histograms in Fig. 3b, the conductances of the channel are 200 and 55 pS, and its reversal potential is -9 mV, which is roughly equal to the nuclear resting potential in the same solution.

Figure 4 summarizes five experiments with 60 mM K⁺/60 mM Na⁺ in the pipette and 120 mM K⁺/0 mM Na⁺ in the bath. The dominate conductance is now 75 pS, and the reversal potential ranges between -21 mV and -15 mV. We assume that this channel corresponds to the 200 pS conductance in Fig. 3, because longer steps fail to show higher conductances (inset in Fig. 4). In addition, channel activity at $V_p = 0$ mV implies there

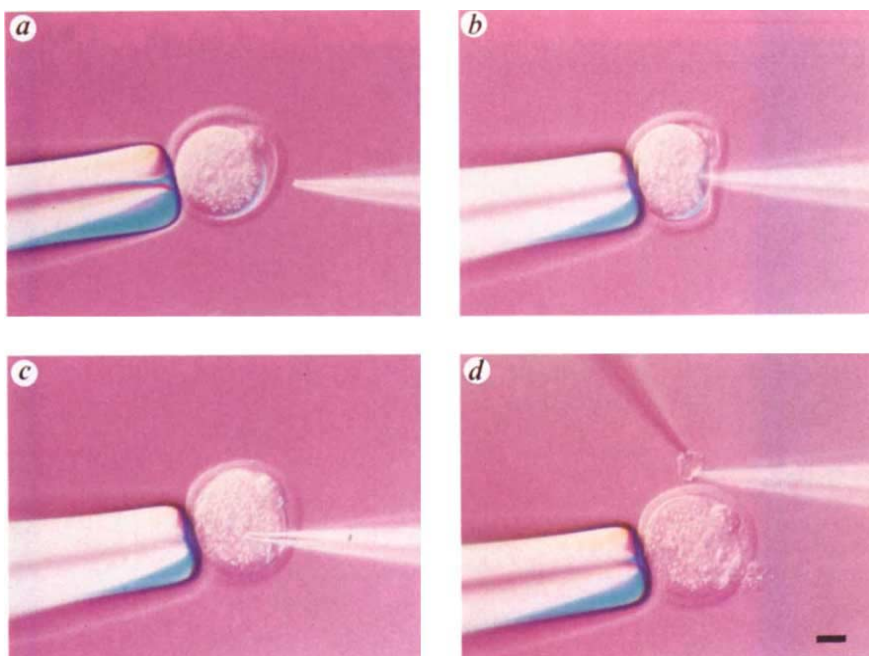
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FIG. 1 A summary of the procedure we used for the transfer of viable pronuclei, adapted to intracellular microelectrode and patch-clamp recording. The scale bar in *d* indicates 20 μm . Female B6D2 F_1 mice were superovulated using 5 IU of pregnant mare serum gonadotropin (Organon, Holland), which was followed in 48 h by 5 IU human choriongonadotropin (Sigma) and individual mating with B6D2 F_1 males. Zygotes were collected ~ 12 h after mating. Cumulus cells were removed from the zygotes by incubation in a 1% hyaluronidase solution for several minutes. After transferring the zygote to the bath solution (in mM: 120 KCl, 2 MgCl_2 , 1.1 EGTA, 0.1 CaCl_2 , 5 glucose, 10 HEPES, pH 7.4), which is a cytoplasmic-like solution used in the intracellular electrode to perform whole-cell experiments on mouse oocytes, we extracted one of the pronuclei using micromanipulation²⁵. The sequence of steps from holding the zygote by the zona with light suction (a), entering the zygote with a smaller suction pipette (b), attaching it to the pronucleus (c), and extracting and holding the pronucleus for patch clamping (d), takes less than 10 min. In *d*, the third pipette on the top is a patch electrode placed on the nuclear envelope surface. We made no attempt to distinguish sperm and oocyte pronuclei in either the resting potential or the single-channel measurements. To measure intracellular and intranuclear membrane potentials, we used an Axoclamp amplifier and glass microelectrodes (70 to 90 megohm, filled with 3 M KCl), which we balanced off with the bridge circuit. The patch electrodes were pulled from hard borosilicate glass (Corning 7052) on a Sachs-Flaming P-84 puller (Sutter Instruments). The pipettes were coated with Sylgard (Dow Corning) and fire-polished to an external tip diameter of ~ 1 – 2 μm . These electrodes had resistances of 4–10 megohms. The estimated surface area of the patch formed with these electrodes is



between 5 and 6 μm^2 . Using a List EP-7 current-to-voltage converter, we applied standard cell-attached patch techniques to obtain 1–10 gigohm seal, nucleus-attached patches from pronuclei. In the single-channel experiments, the pronucleus was bathed in standard 120 mM K^+ cytoplasmic-like solution; the patch pipette contained either this same solution or a 60 mM K^+ /60 mM Na^+ solution. We performed the experiments immediately after extracting the pronucleus, pressing the electrodes against areas of the nuclear membrane that appeared to be clear of cytoplasmic material (d).

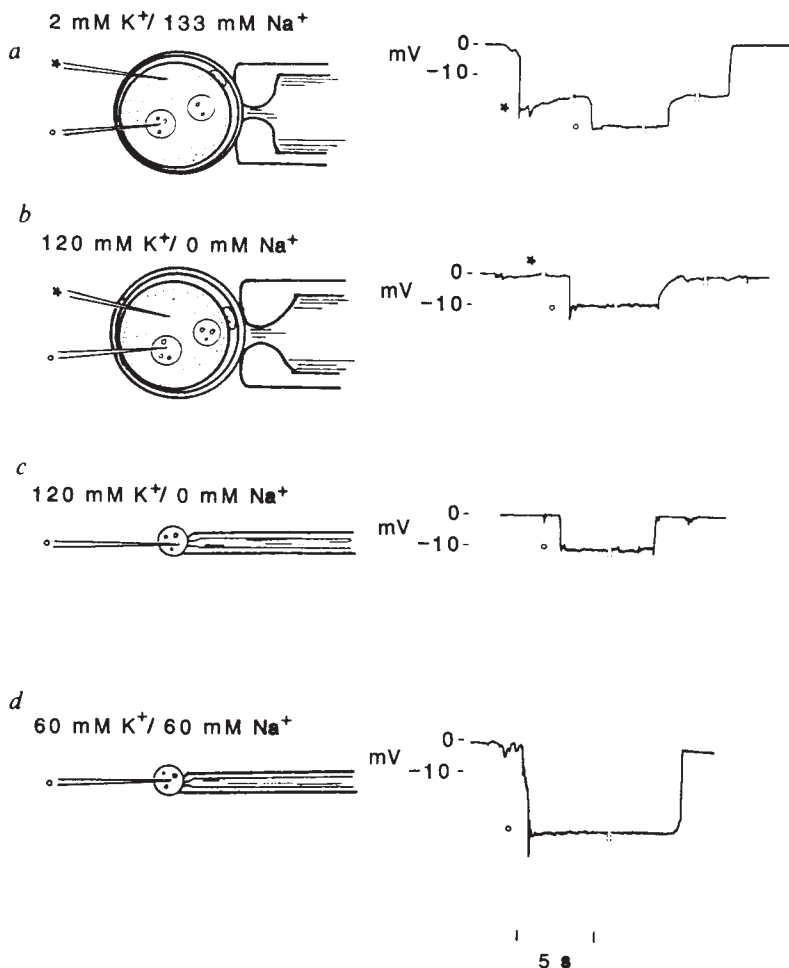


FIG. 2 We performed four types of resting potential experiments. *a*, With the zygote bathed in an extracellular-like solution (in mM: 133 NaCl, 2 KCl, 1.5 CaCl_2 , 0.5 MgCl_2 , 10 HEPES, 5 glucose, pH 7.4), we set the electrode tip potential to zero. We then inserted the microelectrode into the cytoplasm to record the cell membrane potential (*). Under visual control, we then drove the microelectrode into the pronucleus to record the nuclear membrane potential with respect to the cytoplasm (circles). In *b*, we followed this same procedure, now bathing the zygotes in the cytoplasmic-like solution used in the pronuclear extraction (see Fig. 1) to abolish the cell membrane potential. Here we set the electrode tip potential to zero in the cytoplasmic-like solution. In *c*, we directly measured the nuclear membrane potential by extracting the pronucleus and bathing it in the cytoplasmic-like solution (see Fig. 1) and finally, in *d*, we repeated the direct measurement on the pronucleus after replacing the 120 mM K^+ in the cytoplasmic-like solution with 60 mM K^+ /60 mM Na^+ . During measurement of resting potentials, we estimated resistances by rebalancing the bridge after entering the nucleus, which indicated that nuclear resistances are less 1 megohm. We discarded records unless the potentials reversed upon withdrawal. All experiments were at room temperature (26–28 $^{\circ}\text{C}$).

FIG. 3 Single-channel measurements from the nuclear envelope. *a*, Examples of current traces elicited by: +20 and -20 mV, +30 and -30 mV steps. Seal resistances ranged from 1 to 10 gigohms. All traces are leak-subtracted. *b*, Examples of amplitude histograms derived from six concatenated current traces; each of the six traces is from the same experiment and is similar to the example shown to the left of each histogram. Thus a total of 3 s of data went into each histogram at each voltage. We assumed the first peak on each histogram (the dotted vertical line) to represent zero current. The second peak, which is more prominent in the outward direction, is barely visible at $V_p = +20$ (inset) and is unresolved at $V_p = +30$. In *c*, we plot the $i(V)$ relation, using the average nuclear membrane potential, -9.5 mV from protocol *c* (Fig. 2) to find the absolute membrane potential. The open triangles are the currents at the centre of the larger peaks, and the dotted line is a non-linear, least-squares fit to these points. The conductance is ~ 200 pS. Another smaller peak, more evident when the channel conducts outward current, is also plotted (open diamonds). This second smaller peak, with conductance of ~ 55 pS, is evident in the top two histograms, but is generally absent for the inward currents. The blow-up inset for the histogram at $V_p = +20$ mV shows data from a hyperpolarizing potential in which the smaller peak (arrow) is barely visible; the current read from the inset is the only inward current on 55 pS line. In 55 separate experiments, the 200 pS channel appeared in $\sim 10\%$ of the patches; the other patches contained either no channels (10%) or smaller conductance channels (80%), matching the lower conductances that appear when the 200 pS opening was present. Thus, channels are present in nearly every patch, but they are rarely in the highest conductance state. In all single-channel experiments the high-frequency cutoff was set at 1,000 Hz before analysis.

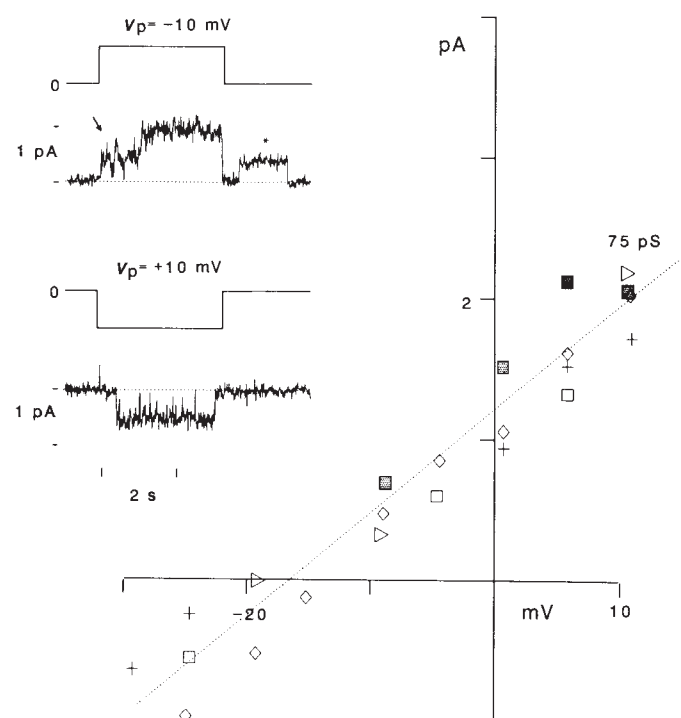
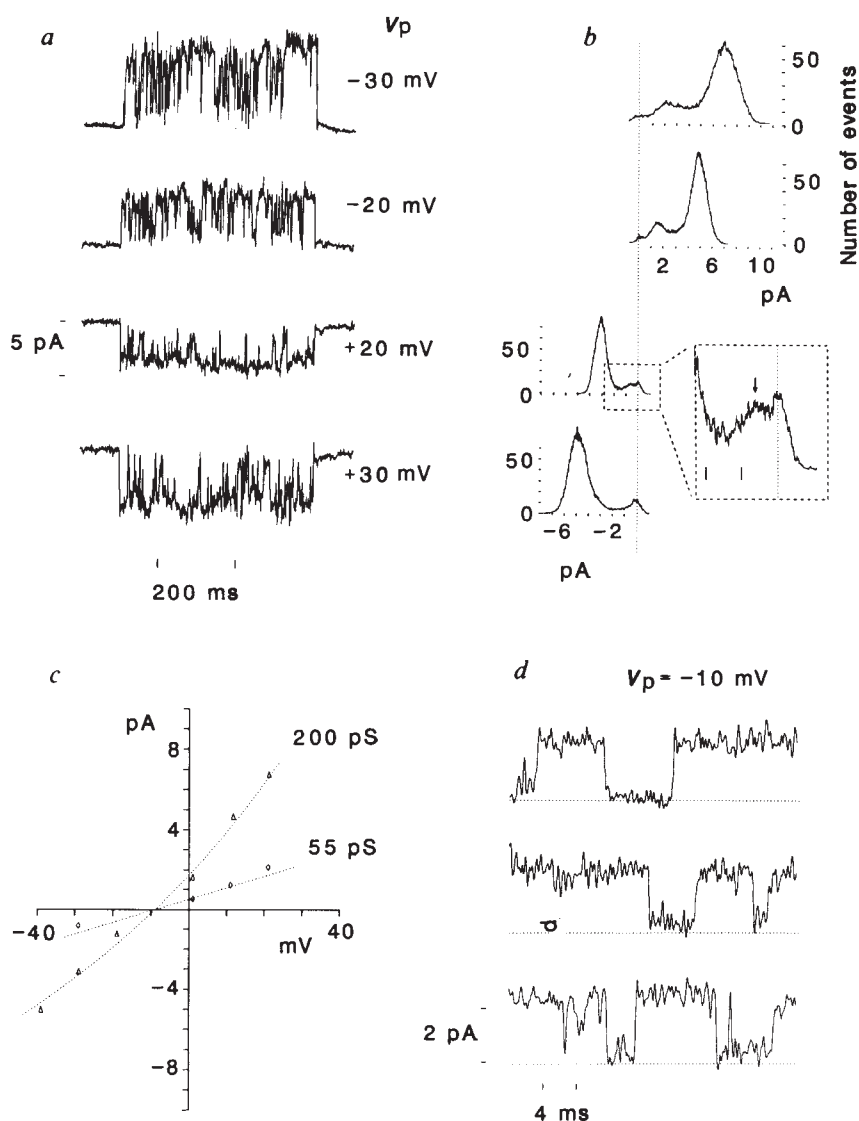


FIG. 4 To investigate channel selectivity, we filled the patch pipette with a 60 mM K^+ /60 mM Na^+ solution while bathing the nucleus in the 120 mM K^+ solution (Figs 1 and 2). The points on the $i(V)$ plot are from five separate nuclei. The current levels were derived from lines drawn through the centres of the largest openings at the different voltages. The voltages are calculated from the equation, $V = (-9.5 - V_p)$. In the upper left quadrant we show examples of two voltage steps and the accompanying current traces at $V_p = -10$ mV and $+10$ mV. The arrow in the top trace indicates conductance levels lower than we used in the $i(V)$ plot. An example of an opening at $V_p = 0$ (*) also appears in the top current trace, something we never see with 120 mM K^+ in the pipette. The points at $V_p = +10$, 0 and -10 mV are plotted as diamonds. The cutoff frequency is 1,000 Hz.

is a driving force in 60 mM K^+ /60 mM Na^+ that we never see in 120 mM K^+ /0 mM Na^+ .

The evidence for a K^+ -selective pathway in the nuclear membrane can be summarized as follows: (1) decreasing the K^+ in the bath of the entire nucleus from 120 mM to 60 mM shifts the nuclear resting potential from -9.5 to -23.5 mV; (2) decreasing the K^+ in the patch from 120 mM to 60 mM shifts the reversal potential of channels in the patch from -9.5 to between -21 and -15 mV; (3) decreasing the K^+ in the patch from 120 to 60 mM also lowers the conductance of the channel from 200 to 75 pS. Assuming that K^+ is 20% more concentrated in the nucleus than in the cytoplasm²⁰, a K^+ -selective membrane would generate -10 mV in 120 mM K^+ and -26 mV in 60 mM K^+ , which are roughly equal to the nuclear potentials in these same solutions. Although -10 mV is near the reversal potential of the nuclear channel in 120 mM K^+ , -26 mV is significantly higher than the reversal potential in 60 mM K^+ , from which we infer that the channels are not exclusively responsible for the nuclear potential.

The nuclear channel is similar in some respects to the K^+ -selective pore in the sarcoplasmic reticulum^{28,29}, which is continuous with the outer membrane of the nuclear envelope, but the sarcoplasmic reticulum channel in neutral bilayers has a conductance of ~ 100 pS in symmetric 120 mM KCl and 50 pS in 120 mM NaCl; also its conductance is linear from -100 mV to 70 mV. Despite these differences, our experimental conditions are too dissimilar to exclude the possibility that the channel we observe in cell-free nuclei is a sarcoplasmic reticulum-like pore.

Electron micrographs of our preparation indicate 3.3 pores per μm^2 , or 4,000 per nucleus. If the channels are the nuclear pores, patch recording greatly underestimates their number. Assuming a pore has a conductance of 1,000 pS (90 Å diameter, 800 Å long³⁰, and containing a solution of 100 ohm . cm), then an upper limit for total nuclear conductance is $4 \mu S$ (0.25 megohm). If 80% of the pores have a conductance of 55 pS and 10%, of 200 pS (Fig. 3), then a lower limit for the total nuclear conductance is $0.25 \mu S$ (4 megohm). We measured 1 megohm or less, and our attempts to voltage-clamp with 70–90 megohm microelectrodes probably failed for this reason. If the pores are not the channels, either there are no pores in our patches, or the pores are closed. This explanation is surprising, because it is contrary to the view that nuclear pores are open holes. Even if the pores are artificially closed in our experiments, we must enquire about the function of the 200 pS channels in the natural state. We speculate that the role of these channels may be to balance the charge carried by macromolecules moving in and out of the nucleus, and therefore they could be involved in regulating gene expression and cell division. □

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A serine protease triad forms the catalytic centre of a triacylglycerol lipase

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TRUE lipases attack triacylglycerols and act at an oil–water interface; they constitute a ubiquitous group of enzymes catalysing a wide variety of reactions, many with industrial potential. But so far the three-dimensional structure has not been reported for any lipase. Here we report the X-ray structure of the *Mucor miehei* triglyceride lipase and describe the atomic model obtained at 3.1 Å resolution and refined to 1.9 Å resolution. It reveals a Ser . . His . . Asp trypsin-like catalytic triad with an active serine buried under a short helical fragment of a long surface loop.

M. miehei triacylglycerol lipase is a single polypeptide chain protein made up of 269 residues (molecular weight of an unmodified chain is 29,472). Isolation and purification methods have already been described^{1,2}. The gene encoding the complete enzyme precursor has been sequenced². A recombinant plasmid was introduced and expressed in *Aspergillus oryzae*³. The purified protein was dissolved in 20 mM Tris-HCl buffer, pH 8.05, to a concentration of 15–16 mg ml⁻¹; we used the hanging drop technique with high concentrations of phosphate buffer (55–75% v/v of saturated solution) to obtain single crystals suitable for X-ray structural study. The crystals have the symmetry of space group $P2_12_12_1$, with the unit cell having dimensions $a = 71.6$ Å, $b = 75.0$ Å and $c = 55.0$ Å. If we assume one molecule per asymmetric unit, the specific volume⁴ is 2.5 Å^3 per dalton.

Table 1 summarizes the crystallographic results. A single major site in the Hg derivative was used to obtain the initial single isomorphous replacement anomalous scattering phases, which allowed the identification of three Pt sites using difference Fourier techniques. The resulting set of phases obtained with two derivatives (mean figure of merit, $\langle m \rangle = 0.58$) was used to identify five principal sites in the iodinated derivative. Subsequent difference Fourier calculations using all three derivatives revealed two other secondary Hg sites and four more iodine sites. Recalculation of phases gave $\langle m \rangle = 0.74$. The initial electron density map calculated at 3.2 Å resolution using only two derivatives (Hg and Pt) was interpretable, although some features of the structure were obscure. A partial model consisting of $\sim 70\%$ of the molecule was constructed and used for a phase combination procedure. At the same time, a third derivative (I) was included in the isomorphous phasing, which led to a substantial improvement in the quality of the multiple isomorphous

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replacement electron density map. Over 75% of the structure was now readily defined. Refinement by restrained least-squares minimization procedures⁵ resulted in an atomic model for which the conventional crystallographic *R* factor was 0.339 at 2.0 Å resolution. Two fragments in particular were poorly defined in the electron density map: the N-terminal fragment comprising the first 13 amino acids, and a long external loop (residues 36 to 48). A polyalanine chain was fitted into the electron density of this loop, and the whole model was refined further, incor-

porating molecular dynamics⁶ to overcome the problem of local minima. This reduced the *R* factor to 0.289 and allowed us to identify correct conformations for most of the remaining residues, with the exception of the 13 N-terminal amino acids. Subsequent manual rebuilding using the program FRODO⁷ and restrained least-squares refinement⁵ improved the geometry of the model, with a final *R* value of 0.249. A subsequent electron density map enabled us to revise part of the loop between residues 36 and 44 and to identify the positions of residues 5

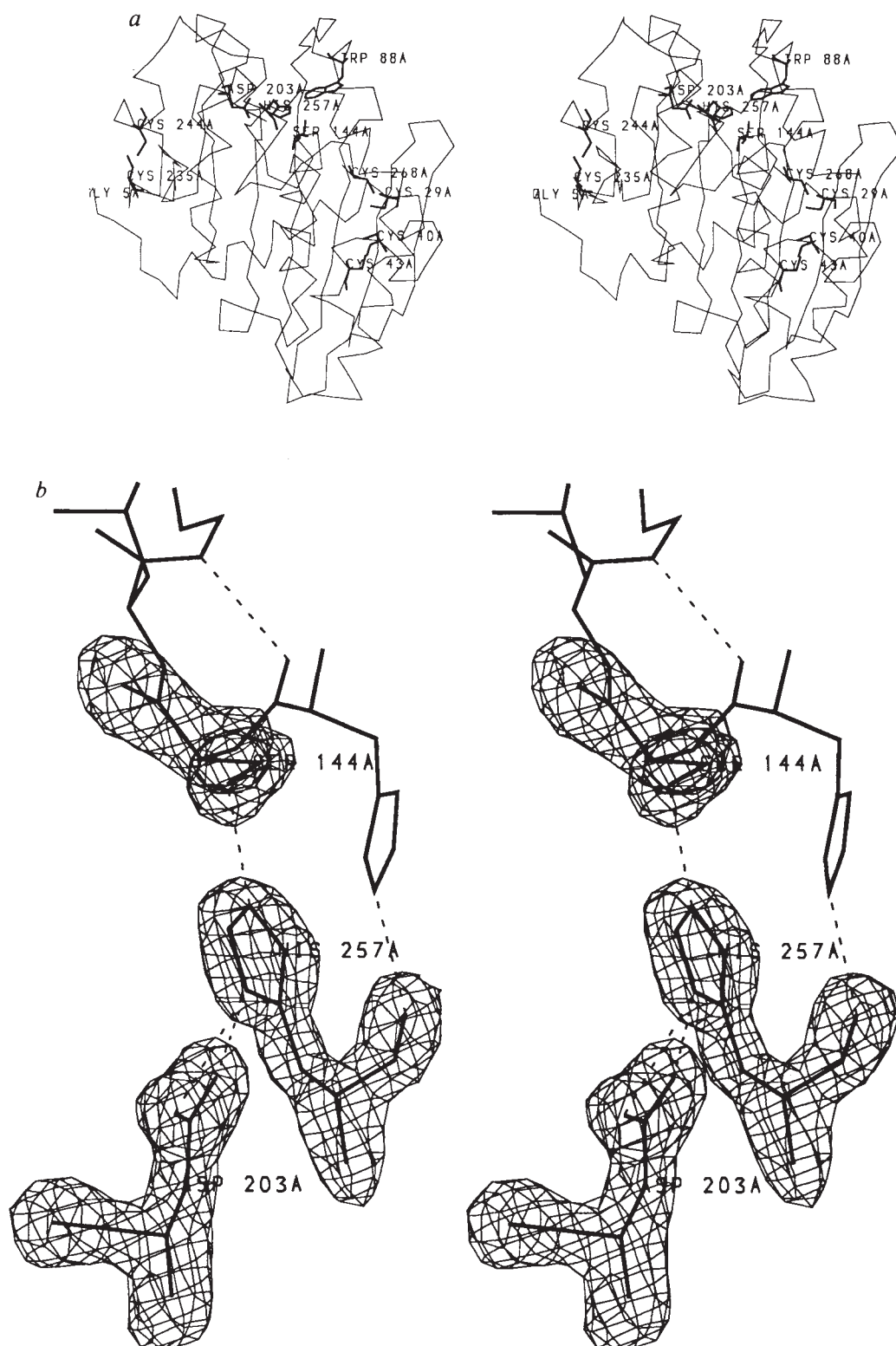


FIG. 1 *a*, Stereo diagram showing the C α plot of *M. miehei* lipase. The three residues of the catalytic triad, the six cysteines that form three disulphide bridges and the Trp 88 residue that constitutes the central part of the lid covering the active serine, are shown. *b*, Stereo figure showing the final difference electron density for the three catalytic amino acids; these have been removed from the structure factor and phase calculation.

to 13. Further refinement calculations, which in the final stages included 233 solvent molecules, are now complete; the value of the *R* factor for all data between 7.5 and 1.9 Å is 0.138 (Table 1).

The *M. miehei* lipase is an α/β type protein, with a central

8-stranded mixed β -pleated sheet folded onto a highly amphipathic N-terminal helix, and a number of loops and helices, including a semi-external, long kinked α -helix (Fig. 1*a*). The central β -pleated sheet is remarkably similar to the β -sheet found in carboxypeptidase A (ref. 8), although in the lipase

FIG. 2 The catalytic triad and the oxyanion hole as observed in *a*, chymotrypsin (entry 4CHA in the Protein Data Bank¹⁸); *b*, subtilisin (entry 1SBC); and *c*, *M. miehei* lipase; the nitrogen atoms participating in the formation of the oxyanion hole are highlighted. Note that the orientation of the oxyanion hole in relation to the triad is different in the three enzymes.

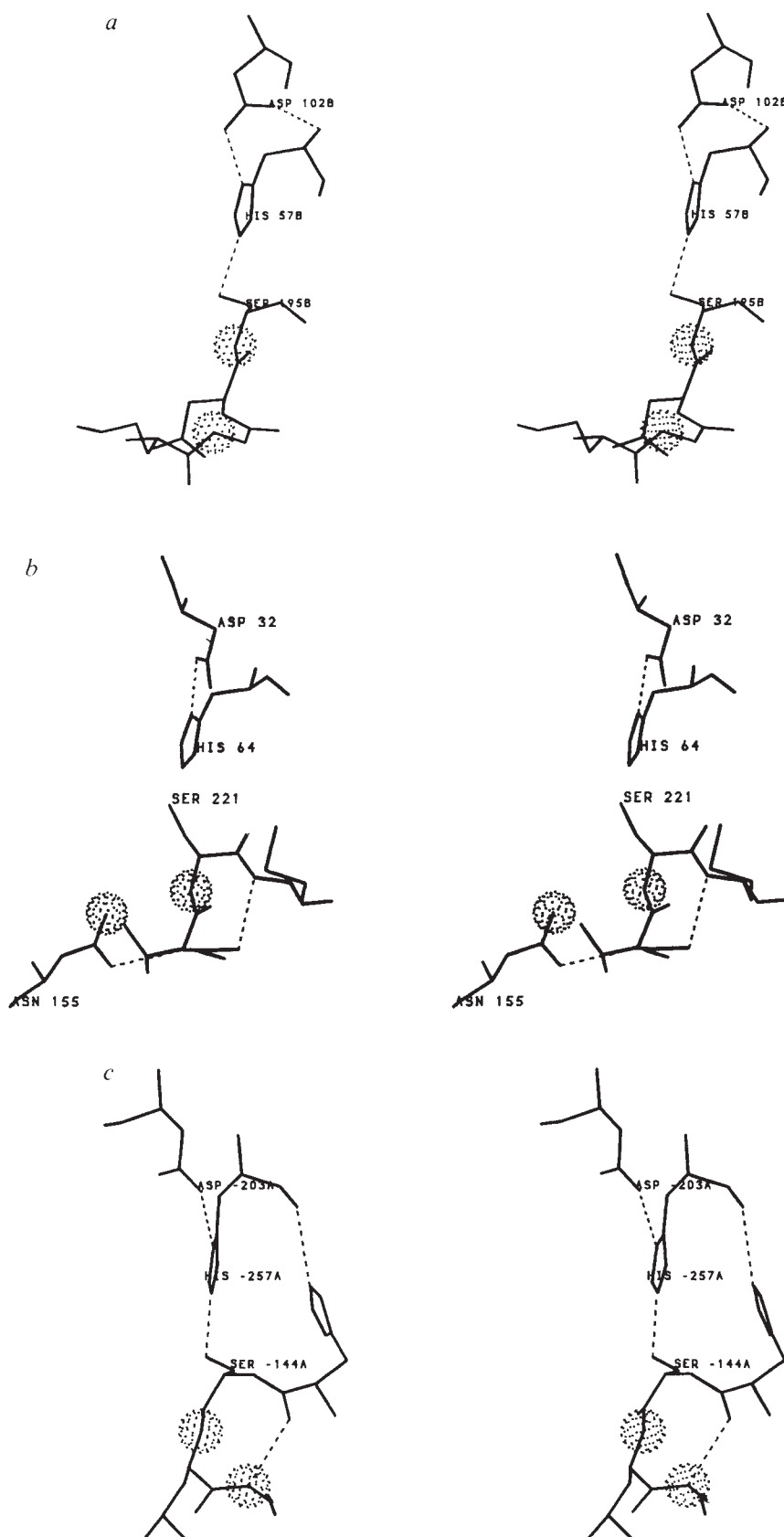


TABLE 1 Summary of crystallographic data

Data set	Native	Hg	Derivative Pt	I
Resolution (Å)	1.9	3.2	3.12	3.11
R_{merge} (%)	8.1	7.8	9.4	7.9
Number of unique reflections	21,165	4,708	4,551	4,862
(% of total possible)	(87.8)	(89.5)	(81.5)	(87.2)
Degree of redundancy	5.5	5.3	6.6	3.4
$\langle I \rangle / \langle \sigma(I) \rangle$	19.9	19.9	19.1	25.2
R_{iso} (%)		20.5	19.3	22.5
R_c (%)		48.9	58.3	48.5
R_{cryst} (%)	13.8*			
Number of heavy metal sites		3	3	9

Data were collected using the Xenotronics area detector mounted on a conventional source ($\lambda = 1.5418 \text{ \AA}$) and positioned 10 cm from the sample¹⁹; heavy atom derivatives were obtained by soaking native crystals as follows: Hg—in 5 mM HgCl_2 for 3 days; Pt—in 2.5 mM K_2PtCl_4 for 2 days; I—in 2.5 mM *N*-iodosuccinimide anhydride for 12 h (details of this new iodination technique will be published elsewhere). Abbreviations: R_c , centric R factor ($\sum(|F_{\text{PH}} - F_{\text{P}}| - |F_{\text{H}}|) / \sum|F_{\text{PH}} - F_{\text{P}}|$, where F_{PH} and F_{P} are the structure factor amplitudes for derivative and native respectively, and F_{H} is the calculated heavy-atom structure factor); R_{iso} , mean fractional isomorphous change; R_{merge} , conventional merging R factor calculated for symmetry-related reflections (on intensities); R_{cryst} , $\sum||F_{\text{O}}| - |F_{\text{C}}|| / \sum|F_{\text{O}}|$ where $|F_{\text{O}}|$ and $|F_{\text{C}}|$ are the observed and calculated structure amplitudes respectively; Degree of redundancy, ratio of total number of observations measured to the number of unique reflections.

* The final round of the restrained crystallographic least-squares refinement⁵ was carried out for 2,055 non-hydrogen protein atoms and 233 water molecules using 19,305 observed data between 7.5 Å and 1.9 Å; the low resolution cutoff was imposed because of the dramatic difference between $\langle F_{\text{O}} \rangle$ and $\langle F_{\text{C}} \rangle$, caused by unaccounted solvent continuum. The current r.m.s. deviation from ideal bond lengths is 0.029 Å, from planarity 0.027 Å and the mean individual isotropic temperature factor (B) is 23.3 Å² (including water molecules).

molecule strand 7 runs in the opposite direction to that in carboxypeptidase A; the r.m.s. difference between the two atomic models (calculated for main-chain atoms only for strands 2–6) is 0.76 Å. This is a dramatic illustration of the structural stability of the twisted β -pleated sheet motif⁹, despite the absence of any sequence homology. There is no similarity between any of the connecting fragments.

Three disulphide bonds have been identified: the first is between cysteines 29 and 268 and stabilizes the global fold; the second is between cysteines 40 and 43, and the third links Cys 235 with Cys 244. This bond could be necessary to stabilize the two terminal strands that extend into solution and are longer than their equivalents in carboxypeptidase A.

We have found a trypsin-like catalytic triad (Ser...His...Asp) in the lipase molecule, which is close to the surface but not completely exposed to solvent. It is buried under the head of a long loop (henceforth referred to as the 'lid') folded onto the triad and stabilized by extensive hydrophobic and electrostatic interactions. The residues forming the catalytic triad in lipase are Ser 144, His 257 and Asp 203. Their side chains form a constellation that is stereochemically very similar to that in serine proteases (Figs 2 and 3). The peptide nitrogens of residues 145 and 146 may be involved in the formation of an oxyanion hole. However, we find no other structural similarities between lipase and the serine proteases.

Lipase sequences from several prokaryotic^{10–12} and eukaryotic^{13–17} organisms are known. It has been shown² that they all contain a Gly-X₁-Ser-X₂-Gly sequence, where X₁ is either Tyr or His; Ser 144 is part of such a sequence in the *M. miehei* lipase. We inactivated the enzyme by labelling it with phenylmethylsulphonyl fluoride (PMSF); subsequent sequence analysis confirmed that Ser 144 is indeed the active residue. But PMSF inactivation of lipase is slow, and further treatment such

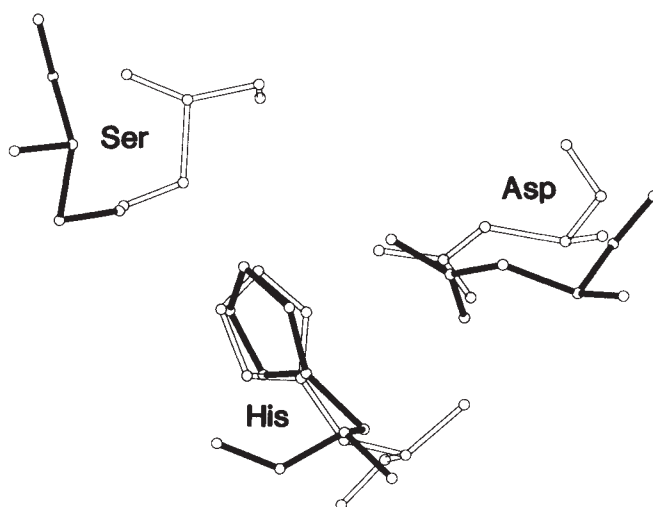


FIG. 3 A comparison of the *Streptomyces griseus* trypsin²⁰ catalytic triad Ser 195...His 57...Asp 105 (open bonds) with that forming the catalytic centre in lipase (full bonds). Residues have been fitted using least-squares and only the atoms from the imidazole ring of histidines, the O_γ of serines and the carboxylic oxygens of the two aspartate residues. Trypsin coordinate set 1SGT (Protein Data Bank¹⁹) was used in this comparison. This view is different from that used in Fig. 2 and is intended to show the conserved stereochemistry of the atoms involved in the activation of serine.

as exposure to a high concentration of 2-propanol or lyophilization is necessary (B. H.-J., unpublished data), consistent with our finding that the active triad is buried under the lid. The enzyme could be working in two stages: the lid is removed or displaced, possibly through interfacial activation, then the ester bond is subsequently hydrolysed by a mechanism very similar to that used by serine proteases. The lid may also serve as a device to inhibit the proteolytic activity of the triad, thereby protecting the enzyme itself.

The atomic coordinates of the catalytic triad and the C_α positions will be deposited with the Protein Data Bank¹⁸. □

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As illustrated in Fig. 3a, the polypeptide chain is clearly divided into two folding units. The larger N-terminal domain comprises residues 1–335 and is typical of an α/β structure dominated by a central parallel β -sheet. The C-terminal domain is of the β -sandwich type and is formed by two layers of β -sheet, each consisting of four antiparallel strands. The central doubly wound sheet¹¹ of the N-terminal domain has nine strands, seven of which run parallel to each other. Five of the six crossover connections between the parallel strands are of the common right-handed $\beta/\alpha/\beta$ or $\beta/\text{loop}/\beta$ type^{12,13}. The last one is the

It has been assumed¹⁴ that porcine pancreatic lipase (PPL) has two functionally important sites, a catalytic site involving an unidentified histidine and a few unknown residues, and a topographically distinct interfacial 'recognition site' controlled by Ser 152. In the structure of HPL this side chain is hydrogen bonded to His 263, which makes another hydrogen bond with Asp 176. Taken together, biochemical and structural evidence indicate that this triad represents the lipolytic site. The triad of HPL has been compared with that of the serine proteases and Fig. 3*b* shows the superposition of the relevant residues after matching five functionally analogous hydrogen bond donor or acceptor atoms (root-mean-square difference, 0.4 Å). Obviously the structural similarity does not extend beyond the positions of the hydrogen bond donors and acceptors. The nitrogen of Gly 193 in trypsin (one of the two donors to the oxanion) is

METHODS. A human pancreatic cDNA library in λ gt11 was screened on duplicate filters with a mixture of antisense oligonucleotides 1 and 5, or 3 and 4, respectively. Oligonucleotides 1 and 3 were designed from identical regions in the porcine⁵ and the canine⁶ pancreatic lipase amino-acid sequences. Oligonucleotide 1, a 23-mer, is 12-fold degenerate with the sequence 5'-TTCCAGTCNACACA(ATG)ATGCAGTT-3', derived from amino acids 100-107. Oligonucleotide 3, a 20-mer, is 16-fold degenerate with the sequence 5'-CCNCCATTNGGGAAGAATC-3' derived from amino acids 225-231. Oligonucleotides 4 and 5 were designed from regions of HPL whose amino-acid sequence had been established from tryptic peptides. Oligonucleotide 4, a 32-fold degenerate 29-mer, has the sequence 5'-TGGAAGTTGTTNNGGTTCTT(GC)ATTNGTGTGA-3' derived from amino acids 41-50 of HPL; oligonucleotide 5 is a 36-fold degenerate 23-mer with the sequence 5'-CCCTCCCA(GTA)ATNCCATC(GTA)ATGTC-3' derived from amino acids 247-254 of HPL. Degeneracies of 2 or 3 nucleotides are given in parentheses. Clones hybridizing to both pools of probes were sequenced by the dideoxy chain termination method.

TABLE 1 Crystallographic data

Space group: $P2_1$ ($a=47.8$ Å, $b=112.8$ Å, $c=91.0$ Å and $\beta=99.3^\circ$)					
Derivative	Native	Mercury acetate	Lead acetate	Uranyl acetate	Mercury/Lead acetate
Number of measurements (number of crystals)	93,075 (3)	49,096 (1)	41,762 (1)	59,197 (4)	42,112 (2)
Number of unique reflections (completeness, %)	33,771 (92)	20,815 (93)	19,397 (87)	20,548 (92)	18,254 (82)
Resolution limit (Å)	2.40	2.83	2.83	2.83	2.83
R_f^*	0.057	0.083	0.061	0.096	0.066
Mean fractional isomorphous change	—	0.15	0.20	0.26	0.24
MIR phasing in resolution range 12.0–2.9 Å: r.m.s. $F_o/E^\dagger$ (all reflections/highest resolution range)		1.7/1.1	2.0/1.7	2.0/1.6	2.2/1.4
R_C (only h01l) ‡		0.60	0.60	0.56	0.54
Number of major/minor sites		2/3	4/1	2/2	6/3
Mean figure of merit	0.72 for 20,655 reflections 0.52 for 2,928 reflections in highest resolution range				

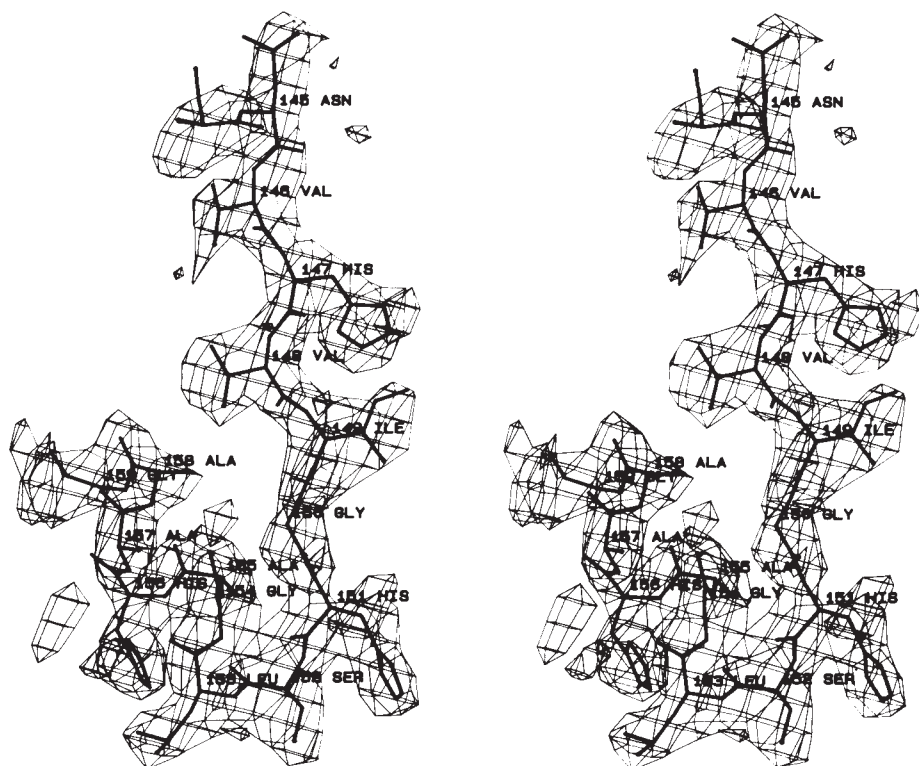
* $R_f = \sum |I - \langle I \rangle| / \sum \langle I \rangle$.

$^\dagger F_o$, heavy atom structure factor; E , residual lack of closure.

‡ Cullis R -factor = $\sum |F_o(\text{obs}) - F_o(\text{calc})| / \sum F_o(\text{obs})$.

The optimized conditions for crystallization of HPL using the hanging-drop vapour-diffusion method were: initial concentrations, 4–8 mg ml⁻¹ protein, 50 mM sodium cacodylate, pH 5.5, 150 mM LiCl, 1% β -octyl glucoside (Calbiochem) and 6–8% (w/v) polyethylene glycol 8000 (Sigma) in a 10 μ l drop equilibrated against a reservoir containing 100 mM sodium cacodylate, pH 5.5, 300 mM LiCl and 12–16% PEG 8000. The presence of LiCl and detergent produces a two-phase system. Single crystals up to a size of $0.30 \times 0.20 \times 0.15$ mm³ can be grown in this way at room temperature within a few days, but there is a strong tendency for crystals to form heavily twinned clusters. All X-ray diffraction data were measured using a Xentronics area detector. The X-ray source was an Elliot GX-21 rotating anode generator operating at 40 kV, 80 mA, with a graphite monochromator. Crystals were cooled to $\sim 4^\circ\text{C}$ during the measurement. Data frames of 0.15 to 0.25°, with exposure times between 120 to 180 s, were collected, mostly by rotation around the unique b axis to optimize the quality of anomalous differences. Data were processed using the XDS software package²³. The merged native data set was used as a reference data set in the frame scaling of the derivative data sets. We used the CCP4 computing package (from the Daresbury Laboratory, UK) for all subsequent crystallographic calculations. Final heavy atom parameters used for phasing were obtained by a Dickerson type refinement²⁴, as implemented in the CCP4 program PHASE. The anomalous differences of the three single derivatives were included in the phasing and were also used to determine the correct enantiomorph.

FIG. 2 Stereo picture of electron density at 2.9 Å resolution using phases after 6 cycles of twofold averaging and solvent flattening. Contours are drawn at 15% of the maximum density value. Residues 144 to 159 of the refined model are shown superimposed.



not closely matched by an equivalent donor in HPL. The closest candidate is the main-chain nitrogen of Phe 77, which is part of a chain segment that may reorientate on activation (see below). A pronounced difference between HPL on the one hand and trypsin on the other is that after superposition, the chain directions at the active site serine are almost antiparallel. As a consequence, N 152 of HPL cannot be equivalent to N 195 of trypsin (the second donor to the oxyanion), instead it is N 153

that occupies the equivalent position. The known hepatic and lipoprotein lipase sequences can be aligned with those of pancreatic lipases¹⁵, and the essential triad residues are conserved in all known sequences. More direct evidence for the proposed active site was provided by a 3.0 Å-resolution data set from a crystal soaked in a solution containing butylboronic acid, a reversible inhibitor of pancreatic lipase^{16,17}: a difference electron density map has its two highest peaks very close to the hydroxyl

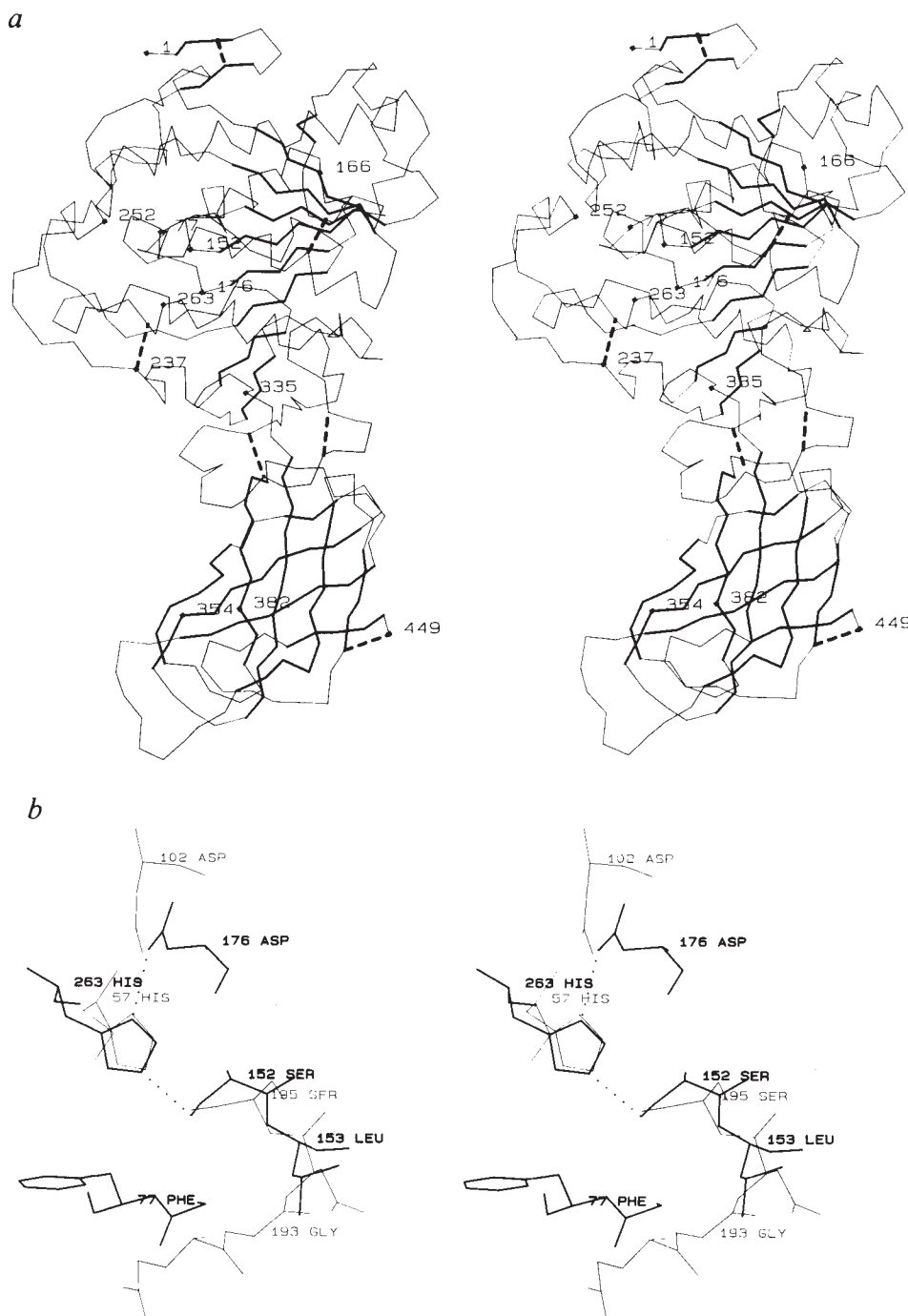
group of Ser 152 in both subunits. Although a tetrahedral boronate adduct has apparently been formed, the occupancy of the inhibitor has turned out to be too low to determine its detailed conformation reliably.

If we accept that the triad with Ser 152 is essential for hydrolysis of the natural water-insoluble substrates, then the presence of another site has to be postulated to explain the hydrolytic activity of PPL when modified at this serine with diethyl-*p*-nitrophenylphosphate; some so-called soluble substrates like *p*-nitrophenylacetate (PNPA) or triacetin can still be hydrolysed at submicellar concentrations⁴. A site that can hydrolyse PNPA (but not triacetin) has been found in the C-terminal domain of PPL, which can be obtained by chymotryptic digestion¹⁸. One of the two histidines present in this C domain of PPL (residue 354 or 382) is likely to be the attacking nucleophile; both these histidines are exposed in HPL. Lys 373 is stoichiometrically

acetylated after prolonged incubation of PPL or of its C-terminal fragment with PNPA¹⁹: as its side chain is next to His 354, it is conceivable that migration of the acetyl group could occur to the lysine from the reactive but labile histidine.

A distinctive feature of the water-soluble lipolytic enzymes is that they hydrolyse substrate efficiently only if it is present as a separate lipid phase. The observed structure of HPL shows that substantial conformational changes must take place before substrate can bind in the postulated active site. It is apparent from Fig. 3a that Ser 152 is completely inaccessible in the structure assumed by the enzyme in the crystal, and probably in an aqueous environment as well. Examination of the chain segments that block access to the active site reveals that the surface loop between the disulphide-bridged residues 237 and 261 covers the active site with a short one-turn α -helix. The repositioning of this loop or flap could be necessary to render

FIG. 3 a, Stereo picture of the α -carbon backbone of human pancreatic lipase. One monomer with its 6 disulphide linkages (4-10, 90-101, 237-261, 285-296, 299-304 and 433-449) drawn as dashed lines between C- α positions is shown. Selected residues are labelled. The β -sheet segments are emphasized by heavier lines. The secondary structure assignments based on the Kabsch-Sanders algorithm²⁵ are for the N-terminal domain: β 1 (2-5), β 2 (9-12), β 3 (37-42), β 4 (50-52), α 1 (56-60), β 5 (69-74), α 2 (85-94), β 6 (99-105), α 3 (114-139), β 7 (146-151), α 4 (153-164), β 8 (171-175), β 9 (198-202), β 10 (224-228), α 5 (248-253), α 6 (261-275), α 7 (288-292), β 11 (323-327); and for the C-terminal domain: β 1 (338-348), β 2 (352-361), β 3 (364-366 and 369-375), β 4 (380-388), β 5 (396-403), β 6 (415-424), β 7 (429-433), β 8 (444-448). b, Stereo picture of a superposition of the Asp-His-Ser triad of human pancreatic lipase (thick lines) with the catalytic triad in the structure of the trypsin benzamidine complex (thin lines)²⁶ deposited as 3PTB in the Brookhaven Data Bank²⁷. The 5 atom pairs OD2 (102/176), ND1 (57/263), NE2 (57/263), OG (195/152) and N (195/153), with residue numbers corresponding to 3PTB and HPL respectively, have been optimally matched in this superposition.



the active site accessible to substrate. When this loop is removed in the model, it is still difficult for a substrate as bulky as a triglyceride to reach Ser 152. Some side chains in segments 75–79 and 212–216 are in intimate contact with the flap, so its removal or replacement by substrate on activation could be accompanied by conformational changes in these chain segments. The bulky Trp 252 is a side chain of the flap which is completely buried and sits directly on top of Ser 152, with one side packed against Phe 77. The functional importance of the flap region is demonstrated by limited tryptic digestion studies of various lipoprotein lipases²⁰. The chief cleavage site is at Arg 228, corresponding to either position 249 or 250 in PPL, according to the alignment in this region. Both positions are exposed to solvent in the HPL structure. Interestingly, the activity of lipoprotein lipase towards soluble substrates is not affected by this cleavage, but it is substantially reduced with emulsions of long-chain acylglycerols and phospholipids²¹. Apparently formation of a productive enzyme–substrate complex at the interface is impeded by this cleavage, consistent with a loop involvement at the interfacial activation step.

A nine-residue segment containing some hydrophobic side chains and the consensus sequence GxSxG has been located in all known mammalian and microbial lipase sequences²². In HPL and PPL, the corresponding sequence is VHVIGHSLG (residues 146–154; one-letter amino-acid code), which contains the active site Ser 152. The GxSxG consensus peptide alone does not represent a significant structural homology; it is also found around the active-site serine in serine proteases and in different structural motifs in other structures. Residues 151–154 form a turn, bringing the two α -carbons of glycines 150 and 154 to within 4.0 Å of each other (Fig. 2); β -carbons on these conserved glycines would collide with the main-chain oxygen atoms of residues 154 and 75 respectively. The predominance of hydrophobic residues in the N-terminal part of the nonapeptide is explained by the packing of these side chains with other hydrophobic side chains; they are certainly not involved in lipid binding²². The questions arise as to whether (1) the conserved serine is the active-site serine in other sequenced lipases and (2) the catalytic triad and its topology are conserved. The three-dimensional structures of other lipases will provide the answers to these questions. □

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Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping

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CYTOMETRIC analysis has identified chromosome 11p13 as the smallest overlap region for deletions found in individuals with WAGR syndrome, which includes Wilms tumour (a recessive childhood nephroblastoma), aniridia, genito-urinary abnormalities and mental retardation¹. The underlying loci have since been resolved into an aniridia (AN2) locus at a telomeric position, and a locus of closely spaced genes or a single pleiotropic gene involved in genito-urinary tract abnormalities and Wilms tumour at a more centromeric position^{2–7}. Pulsed-field gel analysis of the 11p13 region has revealed the presence of several putative CpG islands^{8,9}, structures which are frequently associated with the 5' ends of expressed sequences, mainly housekeeping genes and some tissue-specific genes¹⁰. Starting from a CpG island, we have now isolated four neighbouring CpG islands, all within 650 kilobases (kb), by means of two consecutive bidirectional jumps in rare-cutting restriction-enzyme jumping libraries¹¹. In two instances, flanking sequences were conserved in other species and RNA transcripts were identified. A complementary DNA clone isolated for one of them derives from an RNA highly expressed in fetal kidney, and is predicted to encode a Krüppel-like¹² zinc-finger protein that is probably a transcription factor. The entire cDNA region is included in two partially overlapping homozygous deletions found in Wilms tumour DNA samples. Cloning of the breakpoints in one tumour revealed a deletion size of 170 kb, one-third of which is covered by the cDNA. The expression pattern and sequence of this cDNA could point to an important role for its corresponding gene in the normal development of the renal system as well as in Wilms tumour.

We chose probe 282, located close to the region implicated in Wilms tumour and genito-urinary tract abnormalities (WT-GU region) and near two CpG islands, as a starting point. By chromosome walking, probe A282-EH2.6 containing sites for *Bss*HII, *Sac*II and other rare-cutting enzymes that are not methylated in genomic DNA was isolated. We expected that the 5.1-kb size of the corresponding *Eco*RI fragment would allow jumping in a *Bss*HII–*Eco*RI jumping library in both directions. Screening of ~400,000 clones yielded 11 strongly hybridizing colonies, with the inserts falling into two categories (LFC1 and LFT1), consistent with jumps in both directions (Fig. 1). As chromosome jumping is directional, the type of starting fragment indicated the direction of the jump, which we confirmed by mapping the new end fragments against somatic cell hybrid panels and by PFG analysis to provide correct placement on the pulsed-field (PFG) map (not shown).

To cross the CpG islands, the LFT1 and LFC1 jumping clones were used to isolate lapping λ phage. In the case of LFT1–CP5,

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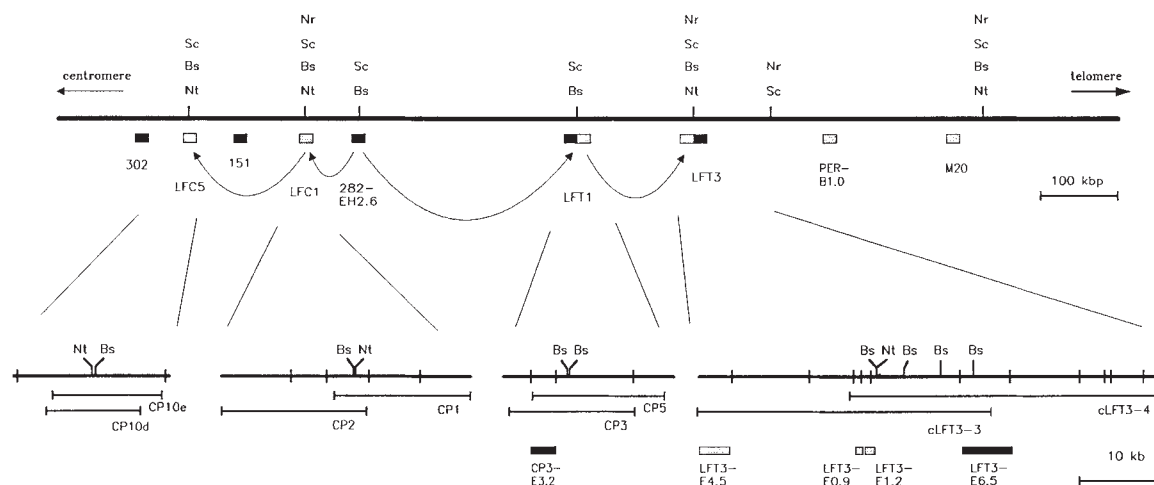


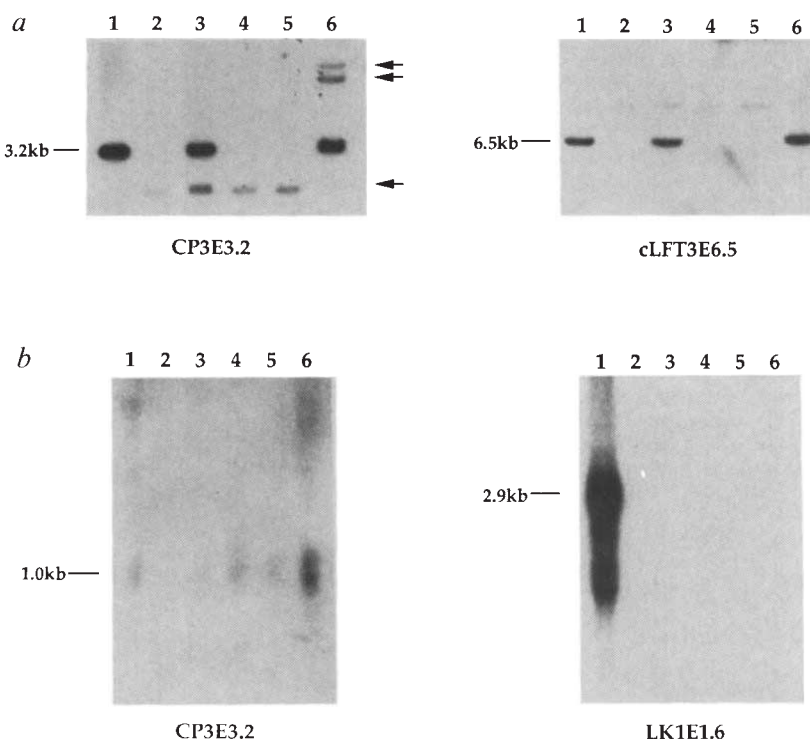
FIG. 1 The WT-GU region: Physical map and chromosome jumping. The PFG restriction map (top) from ref. 9 has been refined to include additional probes. The four directional jumps using probe A282-EH2.6 as a starting point are indicated with previously used random probes shown at their approximate positions. Phage or cosmid clones for the new CpG islands are drawn on a larger scale (bottom). Only two representative clones are shown in each case with their *EcoRI* restriction maps (vertical lines) and cutting sites for *NotI* and *BssHII*. Shaded boxes depict the location of the probes used in Figs 2 and 4; conservation in rodent DNA is indicated by black boxes. Abbreviations: Nr, *NruI*; Sc, *SacII*; Bs, *BssHII*; Nt, *NotI*.

METHODS. Probe A282-EH2.6 was isolated by two chromosome-walking steps in a *MboI* partial digest library in vector EMBL3 from probe 282 (ref. 5). The *BssHII-EcoRI* jumping library¹¹ was plated as a lysogen onto MSI membranes and screened as previously described³⁰. Eleven positive clones were picked and phage induced for re-screening. DNA was prepared from

pure stocks and inserts released by *EcoRI-SalI* cleavage. Fragments likely to represent the anticipated jump were subcloned into plasmid vectors and their position verified by mapping on somatic cell hybrid panels⁵ and comparison of the PFG pattern with that of the starting clone⁹. The new jump fragments were used to isolate longer phage clones from the EMBL3 library and fragments suitable for a consecutive jump were subcloned. The LFC5 and LFT3 jumps were isolated by screening 5,000 phage from a *BssHII/BamHI-HindIII* jumping library with a 1.6-kb *BssHII-HindIII* fragment from CP1 and a 200-bp *BssHII-HindIII* subclone of CP5, respectively. Correct inserts were again used to isolate overlapping EMBL3 or cosmid clones (*MboI* partial digest human placental library in pWE15 provided by G. Evans). Restriction maps for these clones were derived from hybridizations of overlapping clones and by partial digest strategies. All subcloning and hybridization was done according to standard procedures³¹.

FIG. 2 CpG island probes are conserved and contain expressed sequences. *a*, Subclones close to the CpG island map to the 11p13–11p14 region and cross-hybridize with rodent DNA (arrows). *b*, These probes also detect RNA transcripts in fetal RNA (1, kidney; 2, heart; 3, thymus; 4, liver; 5, lung; 6, brain). The CP3E3.2 transcript is expressed in all tissues. Expression of the LFT3-associated transcript was only observed in fetal kidney.

METHODS. *a*, Somatic cell hybrid panels (*EcoRI*-digested human DNA (1), hamster DNA (2), human hamster hybrid DNA containing a normal chromosome 11 (3) or WAGR deletion chromosomes (4,5), and human mouse hybrid DNA (6) containing 11pter-q23) described in ref. 5 were hybridized and washed under normal stringency. Bands in hamster (lanes 2–5) and mouse (lane 6) DNA are marked by arrows. *b*, Fetal RNAs (20–22 weeks) were prepared by the GuSCN-CsCl₂ method, 10 µg each separated on a 1.1% agarose-formaldehyde gel and transferred to Genatran membrane. Hybridization was performed under standard conditions (50% formamide, 5 × SSC, 42 °C) with washing in 0.5 × SSC, 0.5% SDS at 55 °C followed by overnight exposure. A 600-base pair (bp) *Avall-HincII* fragment of CP3E3.2 and the 1.6-kb *EcoRI* fragment of the LK1 cDNA clone were used as probes. Size estimates were derived from the positions of ribosomal RNA and a BRL RNA ladder.



the large size of the telomeric *Bss*HII-*Eco*RI fragment (~10 kb) precluded the use of the same *Bss*HII-*Eco*RI jumping library for a consecutive jump. A second *Bss*HII jumping library, recut with the enzymes *Hind*III and *Bam*HI instead of *Eco*RI, circumvented this problem¹³. Two identical clones (LFT3) from this library mapped to the correct chromosomal position (Fig. 1). Screening of a human cosmid library yielded four overlapping clones, spanning ~60 kb. Hybridization with probes from these cosmid clones confirmed their expected position on the PFG map. We used the *Bss*HII/*Bam*HI-*Hind*III jumping library to

isolate another jump clone (LFC5), centromeric to LFC1 (Fig. 1). Again, overlapping λ clones (CP10) were isolated to encompass the entire CpG island. We therefore isolated four new CpG islands together with their flanking genomic regions.

Several genomic fragments on each side of every CpG island were assayed for cross-hybridization with rodent DNA on somatic cell hybrid panels under high stringency. At two of the four CpG islands we detected conserved fragments (Fig. 2a). On northern blots, a subclone of CP3E3.2 detected a transcript of ~1 kb (Fig. 2b) expressed in all fetal tissues examined,

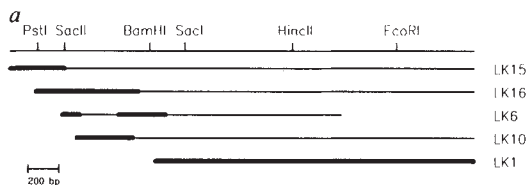


FIG. 3 Sequence of the LK cDNA clones and the zinc-finger motif. *a*, Restriction map of cDNA isolates and sequencing strategy. The clones used for sequence analysis are shown with sequenced portions represented by thick lines. *b*, Nucleotide sequence of the LK cDNAs with the deduced amino-acid sequence (single-letter code) for the open reading frame. The proline-rich regions are located in the N-terminal half of the sequence. The zinc-finger domain extends from nucleotides 1,329 to 1,694, followed by a translation termination codon at position 1,728. The 3' untranslated region contains a simple repeat element (G-T)_n (nucleotides 2,751–2,793) and a polyadenylation signal A-T-T-A-A-A (2,985–2,990), followed by a 16-nucleotide poly(A) tract in LK1. *c*, Deduced amino-acid sequence (single-letter code) of the putative LK-associated zinc fingers, consensus sequence and comparison with the similar *Krox-20*-EGR2 consensus sequence^{15,16}. Capital letters indicate strict conservation, other conserved amino acids are printed in lower-case letters. Boxes indicate the His-Cys link (H-C)³² and amino acids strictly conserved in the *Drosophila Krüppel* gene product.

METHODS. A cDNA library was prepared from 3 μ g fetal kidney poly(A) RNA (20–22 weeks) using the Invitrogen librarian kit. After size selection of the cDNA (>1.5 kb), ligation into λ gt10 and packaging using Gigapack gold (Stratagene) 2×10^6 clones were obtained. Screening of part of the library with a 3-kb LFT3E6.5 subfragment, competed with excess sheared human placental DNA, led to the isolation of six cDNA clones. Re-screening with these cDNA clones resulted in isolation of a total of 15 clones with consistent restriction maps. The longest clone (LK15) is 3 kb. The entire cDNA maps to the 11p13 region in the WT PER-8A deletion area (Fig. 4). Sequence analysis was performed on plasmid subclones using the USB Sequenase kit. Both strands were sequenced. Compressions in the 5' GC-rich regions were resolved by substituting deaza-GTP for GTP. The UWGCG program package was employed for sequence editing and comparison. The zinc-finger domain is underlined and the polyadenylation signal is overlined.

b

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TCAAGGACAGCGCCACACCGGGGGCTCTCCGAAACCCGACCGCTGTCCGCTCCCCCACTTCCCGCCCTCC
74
S R Q R P H P G A L R N P T A C P L P H F P P S
24
CTCCCACTACTCATTACCCACCCACCCACCCAGAGCCGGGACGGCAGCCAGGCGCCGGCCCGCCCGCT
146
L P P T H S P T P R A G T A A Q A G P P R R
48
CTCCTCGCGCGATCCTGAGCTTCTGCTGTCAGGACCGGCTTCCAGCTGTCTCCCGGACCGCGCTCT
218
L L A A I L D F L L L Q D P A S T C V P E P A S
72
CAGCAGCGCTCCGCTCCGGGCTGGTGCCTACAGCAGCAGCAGCAGGAGTCCGGGACCCGGCGGC
290
Q H T L R S G G P G C L Q Q P E Q Q G V R D P F G
96
ATCTGGGCAAGTTAGCGCGCCGAGGCGGAGCGCTGAACGTCTCCAGGCGCGGAGGAGCGCGCGCTCC
362
I W A K L G A A E A S A E R L Q G R R S R G A S
120
GGGTCTGAGCTCAGCAATGGGCTCCGACGTCGGGACCTGAACGGCTGTGCGCGCGCTCCCTCCCTG
434
G S E P Q Q M G S D V R D L N A L L P A V P S L
144
GGTGGCGCGCGCTGTGCTGCTGTGAGCGCGCGCGCAGTGGCGCGCGTGTGCTGCTGCTGCTGCTG
508
G G G G G C A L P V S G A A Q W A P L D F A P
168
CGCGCGCTTCCGCTTACGGGTGCTTGGCGCGCGCGCGCGCGCGCGCTCCGCGCGCGCGCGCGCG
578
P G A S A Y G S L G G P A P P P A P P P P P P
192
CGGCTCAGCTTCTCATAAACAGGAGCGGAGCTGGCGCGCGCGGAGCGCGCAGGAGCAGTCCCTGAGC
650
P P H S F I K Q E P S W G G A E P H E E Q C L S
216
GGCTTCACTGTCCAGTTTCCGGGACCTTCACTGGCAGCGGAGCTGTCCGCTACGCGCGCTTCCGCTCT
722
A F T V H F S G Q F T G T A G A C R Y G P F G P
240
CTCCCGCCAGCCAGCGGTCTATCCGGGACCGGATGTTTCTTAAAGGAGTGTGCTGCTGCTGCTGCTG
794
P P P S Q A S S G Q A R M F P N A P Y L P S C L
264
GAGAGCCAGCCGCTATTGCAATCAGGTTACAGCAGCTCAGCTTGCAGCGGAGCGCGCAGTACGCTCAC
866
E S Q P A I R N Q G Y S T V T F D G T P S Y H
288
ACGCGCTCGGACCATCGCGGCGAGTCCCGACCACTCATTCAAGCATGAGCATCCATGGGCGAGCAGGCG
938
T P S H A A Q F P N H S F K H E D P M G Q G
312
TCGCTGGTGAGCAGCAGTACTCGTGGCGCGCGCTGTATGGCTGCCACACCCCGCAGCAGTGCACC
1010
S L G E Q Q Y S V P P P V Y G C H T P T D S C T
336
GGCAGCGAGGCTTGTGCTGAGGACCGCTTACAGCAGTGAACATTTATACCAATGACATCCGAGCTTAA
1082
G S Q A L L L R T P Y S S D N L Y Q M T S Q L E
360
TGCATGAGTGAATCAGATGAACATAGGAGCCACCTTAAAGGAGTGTGCTGCTGCTGCTGCTGCTG
1154
C M T W N Q M N L G A T L K G V A A G S S S S V
384
AAATGGACAGAGGCGAGAGCAGCAGCAGGAGTACGAGAGGATAACACCAACAGCCCATCTCTGCG
1226
K W T E G Q C A L N P V S G A A Q W A P L D F A P
408
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1298
G A Q Y R I H T H G V F R G I Q D V R V P G V
432
GCCCGACTTGTGATCGGTGCGCATCTGAGCAGCTGAGAAACGCCCTTCTATGTGCTTACCCAGGCTGC
1370
A P T L V R S A S E T S E K R P F M C A Y P G G
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AATAAGAGATTTTAAAGTGTCCCACTTACAGATGACAGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG
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N K R Y F K L S H L Q M H S R K H T G E C K P Y Q
480
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1514
C D F K D C E R R F S R S D Q L K R H Q R R H T
504
GGTGTGAACCATTCAGTGTAAACCTTGTACGGAAGTTTCTCCGGTCCGACCACTGGAAGACCCACAG
1586
G V K P F Q K T C Q R K F S R S D Q L K R H Q R R H
528
AGGACTATACAGGTAAGAAAGTGTAAAGGCGCTTCAAGTGTGCGGTGCGGAGGAGTGTGTCAGAAAG
1658
B T H T G K T S E K P F S C R W P S C Q K K F A
552
CGGTGAGTAAATAGTCCGCGATCACAACATGCATCAGAGAAACATGACCAAACTCCAGCTGCGGCTTGA
1730
R S D E L V R H H N M H Q R N M T K L Q L A L *
576
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1802
ACTGCTGCTCACTAAAGGAAAGTTCAGTTGATCTTCTTCACTCAAACTGCAAGAGCAAGATACCGGTG
1874
CTGGAATACAGAGGTTGCTGCTGAAGAGTGTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
1946
GAGAAGCAGCTAACAAATGTCTGTTAGTAAAGGCCATTGCCATTTGCTGTGATTTTCTACTGTAAAG
2018
AGCCATAGTGTATCATGTCCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
2090
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2306
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2378
ACCATTGATTAATCAATGTGAACACTGGCAGCTGCTGTAAAGAACTATGAAGATCTGAGATTTTGTG
2450
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2522
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2594
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2666
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2882
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2954
TTGTAACCTGCATTTTCACTTGTGCTCAATTTAAAGTCTATTCAAAAGGAAAAAAGAAAAAAGAAAAA
3020

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c

443	T	S	E	K	R	P	F	H	C	A	Y	P	G	C	N	K	R	Y	F	K	L	S	H	L	Q	M	H	S	R	K	H	
474	T	G	E	K	P	Y	Q	C	D	F	K	D	C	E	R	R	F	S	R	S	D	Q	L	K	R	H	Q	R	R	H		
504	T	G	V	K	P	F	Q	C	K	T	-	-	C	Q	R	K	F	S	R	S	D	H	L	K	T	H	T	R	T	H		
532	T	G	K	T	S	E	K	P	F	S	C	R	W	P	S	C	Q	K	K	F	A	R	S	D	E	L	V	R	H	N	M	H
	T	^g _s	e	k	p	f	x	c	x	x	x	x	c	x	^r _k	f	x	r	s	d	x	L	x	x	x	x	x	x	x			
	T	G	X	K	P	F	X	C	X	X	X	X	C	X	R	S	R	S	D	E	L	T	R	H	I	R	I	H				

consensus LK 15
consensus Krox-20

consistent with the characteristics of a housekeeping gene. With the 6.5-kb *EcoRI* fragment of cLFT-6, a hybridization signal was seen only with fetal kidney RNA (Fig. 2b) among the tissues examined. We investigated this transcript further, because it maps in the WT-GU subregion of the WAGR locus.

A total of 15 cDNA clones for the LFT3-associated transcript were isolated from a human fetal kidney cDNA library. The longest clone (3 kb), designated LK15 (Fig. 3a), is similar in size to the RNA transcript, estimated to be 2.9 kb. The transcript probably originates from the CpG island, as the 5' end of LK15 maps within 4 kb of the island. Analysis of genomic DNA and overlapping cosmid clones indicated that the corresponding locus is spread over ~60 kb.

Sequence analysis of the cDNA clones revealed a combined length of 3,020 nucleotides with a single long open reading frame of 1,725 nucleotides starting at the 5' end of LK15 (Fig. 3b). The first ATG codons (positions 381 and 759) in the cDNA sequence do not fit the consensus sequence for initiation codons¹⁴. This observation, taken together with that of an open reading frame extending to the end of the clone, implies that the translation start site of the messenger RNA has not yet been cloned.

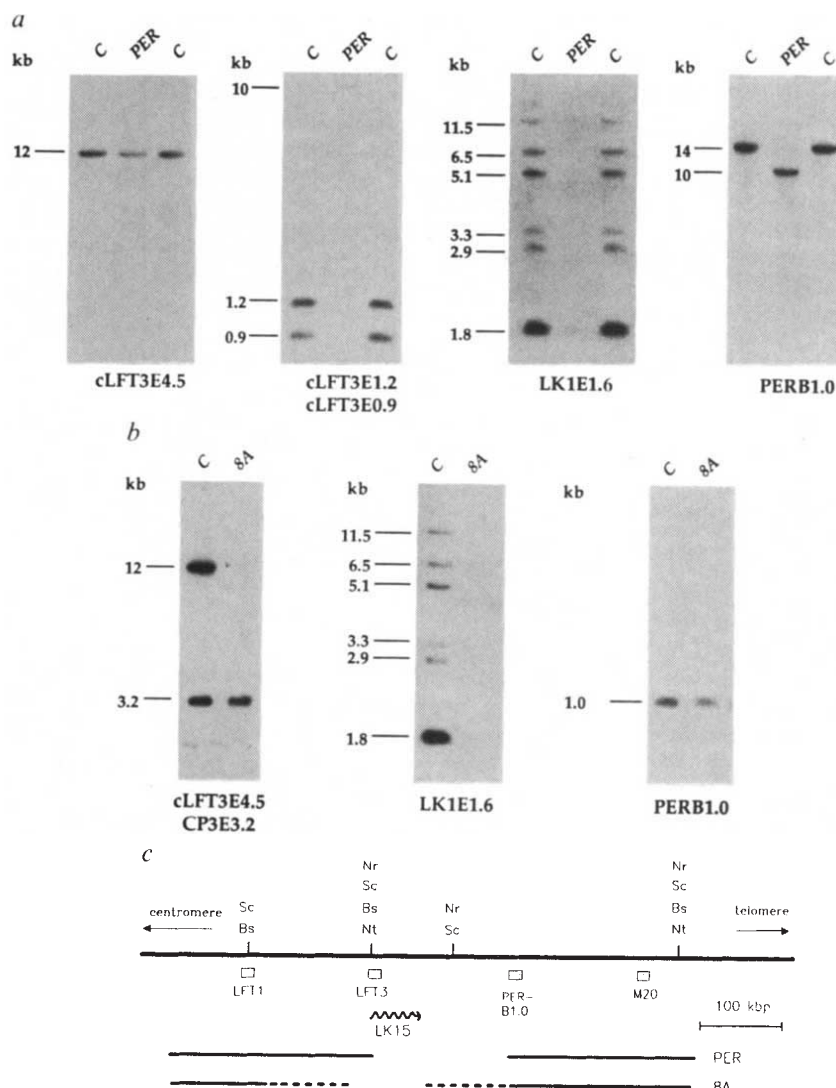
The predicted protein contains four tandemly repeated sequence motifs (Fig. 3c) related to the zinc-fingers of the product of *Krüppel*, a *Drosophila* segmentation gene¹². By sequence comparison, the murine *Krox-20*-encoded polypeptide and its human homolog EGR2 were most similar to that encoded by clone LK15 with 63.1% amino-acid identity in the zinc-finger

domain^{15,16}. *Krox-20* is an early growth response gene activated during G0-G1 transition in cultured cells¹⁵.

Another feature of the translated cDNA sequence is the unusually high proline content, with two regions between amino acids 5 and 46 and 153 and 209 containing 33% proline. The latter region includes a stretch of nine prolines, reminiscent of the cluster of seven prolines near the N terminus of the protein encoded by *Krox-20*¹⁵. Proline-rich regions have also been found in several other putative DNA-binding proteins¹⁷, including the *Krüppel* gene product, and could represent transcriptional activation domains as described for the CTF/NF-1 family of closely related polypeptides¹⁷. From these structural features, it is reasonable to infer that the product of the LK15 cDNA clone is a DNA-binding protein that could be a transcriptional regulator.

We also tested all new CpG islands against a panel of DNAs from 65 Wilms tumours to detect possible deletions or rearrangements. The centromeric clones as well as LFT1 detected no homozygous deletions on Southern blots. But, the telomeric LFT3 probe identified homozygous deletions in two tumours (Fig. 4a, b). Both deletions start between the LFT1 and LFT3 CpG islands and encompass all fragments detected by the LK15 cDNA clone, but do not include the more distal probe M20 (Fig. 4c). The proximal breakpoint in tumour PER is located only ~2 kb centromeric to the CpG island on the 500-kb *NotI* fragment. We cloned the rearranged *EcoRI* fragment of 10 kb and mapped the PER B1.0 subclone to the 375-kb *NotI* fragment between the LK15 cDNA and probe M20. More detailed

FIG. 4 The LFT3-LK1 region is homozygously deleted in two Wilms tumours. *a*, Probe LFT3E4.5 detects a band in DNA from Wilms tumour PER, whereas Probe E0.9 detects a fragment of altered size, and the adjacent probe E1.2 is homozygously deleted. All bands detected by the LK1 cDNA are also deleted. The PER B1.0 probe, isolated from the other side of the cloned deletion junction, detects the same rearranged band in PER DNA as probe E0.9. *b*, In Wilms tumour 8A, the proximal boundary of the homozygous deletion region is located between probes CP5E3.2 and probe LFT3E4.5. The entire LK1 cDNA region including the 3' end (not shown) is deleted in this DNA. The PER B1.0 probe, however, is not included in the homozygous deletion. The extremely weak 1.8 kb band in both PER and 8A that is seen with LK1E1.6 is probably due to a small percentage of normal cells in the tumour samples. *c*, Schematic representation of the extent of homozygous deletions in tumours PER and 8A. Dashed lines indicate uncertainty of the exact breakpoint location in 8A. The extent of the LK15 locus is derived from the analysis of additional overlapping cosmid clones (not shown). The size of the PER deletion was measured by PFG mapping studies not shown here. METHODS. DNA (4 µg) from tumours and normal control DNA (C) were digested with *EcoRI* (or *BamHI* for probe PER B1.0 in *b*), and filters were prepared and hybridized as described⁵. Before using filters again they were stripped in 10⁻³ M Tris-HCl, pH 8, 10⁻⁴ M disodium EDTA, 0.1% SDS at 75 °C. To clone the rearranged fragment in PER, *EcoRI*-digested tumour DNA was ligated into EMBL4 and packaged using Gigapack gold (Stragagene). Screening with LFT3E0.9 gave eight independent isolates. After restriction mapping of the 10-kb deletion junction, suitable fragments were subcloned into plasmid vectors. The PER B1.0 clone correctly maps to chromosome 11p13 and is located ~170 kb telomeric to the LFT3 CpG island as determined by PFG analysis. Abbreviations as in Fig. 1.



mapping (not shown) revealed a deletion size of ~170 kb with both alleles having apparently identical deletions.

The homozygous deletion in Wilms tumour 8A terminates proximal to that in PER, as is also the case for the WiT-13 deletion that includes the CpG island and part of the 375-kb *NotI* fragment^{18,19}. The distal end of the Wilms tumour 8A deletion has not been identified but must be located in the PER deletion. The two deletions therefore overlap by <170 kb, with 60 kb covered by the LK15 locus (Fig. 4c). It seems quite feasible to isolate the remaining region of overlap of both homozygous deletions to determine whether other genes adjacent to LK15 play a part in the development of the genito-urinary system or in Wilms tumour.

The low incidence of homozygous deletions that are detected by our probes, which can be at most 50 kb away from a Wilms tumour locus in this region, and the presence of an apparently normal-sized transcript for LK15 in 12 tumours (data not shown), contrasts with the frequent deletions and internal rearrangements of *Rb-1*, another tumour suppressor gene, seen in retinoblastomas and other tumours²⁰⁻²⁴. It is possible that the presence of another important gene in this region favours more-subtle alterations in these tumours, or loci at other sites in the genome²⁵⁻²⁹ could have a larger role in sporadic Wilms tumours than previously believed.

The expression pattern of the LK15 transcript indicates that its corresponding gene could be involved in normal kidney development, especially because the genito-urinary anomaly component of the WAGR syndrome maps to this genomic region^{6,7}. The presence of the Krüppel-like¹² zinc-finger domain in the LK15 cDNA and its potential function as a regulatory protein is consistent with this model.

Our identification of this zinc-finger gene demonstrates the power of the combined approach of mapping CpG islands by PFG analysis and subsequent cloning by consecutive jumps from one island to the next using rare-cutting restriction-enzyme jumping libraries. Molecular probes for island-associated genes should access a significant fraction of expressed sequences, and, especially in chromosomal regions rich in CpG islands, provide a framework for a gene map. □

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Silver staining of proteins and DNA

C. R. Merril

Silver stains offer high sensitivity for the detection of proteins and DNA separated on gels and membranes. These stains depend on the reduction of ionic to metallic silver.

ONE of the most renowned alchemists, Count Albert von Bollstadt, reported as early as the 13th century that silver could be used to stain organic matter, including human skin. However, it was not until the 19th century that silver staining was used scientifically for histological studies. More recently, silver stains have been adapted for the detection of electrophoretically separated proteins and DNA¹. They have proved to be around 100-fold more sensitive than the most sensitive organic protein stains, and around three times more sensitive than ethidium bromide for the detection of DNA.

Silver staining protocols

The detection of a protein or nucleic acid by silver stains requires the reduction of silver ions to form metallic silver images. This process depends upon differences in redox potential between sites occupied by the biomolecule and surrounding unoccupied sites in the matrix. If the site containing the biomolecule has a higher reducing potential than surrounding sites, the protein will be positively stained. Conversely, if the occupied site has the lower reducing potential, the protein will appear negatively stained. It is possible to alter redox potentials by changing the chemistry of the staining procedure².

Silver stain protocols fall into three broad categories^{1,3}: diamine or ammoniacal stains, non-diamine chemical reduction stains, and stains based on photo-development. The diamine or ammoniacal stains have been designed to release ions in a controlled manner to facilitate the selective reduction of metallic silver. The silver ion concentration is maintained at a low level in these stains by the formation of silver diamine complexes with ammonium hydroxide. Image development is initiated by acidification of the ammoniacal silver solution, usually with citric acid in the presence of formaldehyde. The addition of citric acid lowers the concentration of free ammonium ions, resulting in the liberation of silver ions for reduction by formaldehyde to metallic silver. The diamine or ammoniacal silver stains have proved to be particularly useful for staining proteins separated on polyacrylamide gels that are thicker than 1 mm. Their use in clinical studies (Fig. 1) has facilitated the discovery of specific protein variations in disease states⁴.

Non-diamine chemical reduction stains use silver nitrate to provide silver ions for reaction with protein and nucleic acid sites

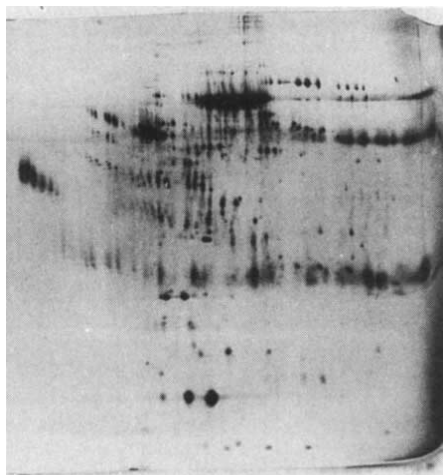


FIG. 1 Over 250 proteins can be visualized with silver staining of this high-resolution two-dimensional electrophoretogram of 40 μ l of unconcentrated human cerebrospinal fluid.

under acidic conditions. Image development is initiated with an alkaline solution, while silver ions are selectively reduced to metallic silver with formaldehyde. The solution must remain alkaline and buffer the formic acid produced by oxidation of the formaldehyde. Non-diamine staining procedures are quick and simple to perform. These stains are better for gels that are less than 1 mm in thickness.

Photo-development silver staining depends on light energy to reduce ionic to metallic silver, and is the basis of the photographic process. Proteins enhance the photo-reduction of ionic to metallic silver in gels impregnated with silver chloride. Photo-development stains are rapid and simple, allowing the visualization of protein patterns within 10 minutes of the electrophoretic separation. However, the current photo-development protocols lack the sensitivity of other silver staining methods and give poor image presentation. They should be reserved for studies of dense protein bands or spots⁵.

Quantitative applications

Most silver stain protocols provide a reproducible relationship between silver stain density and protein and nucleic acid concentration over a 40-fold range in concentration. However, the density-concentration relationship is protein specific. Such a relationship has also been found with Coomassie blue staining and the Lowry protein assay. It probably results

from the different numbers of reactive groups in different proteins — the principal reactive groups being the free amines and the sulphur groups contained in the proteins^{6,7}. Studies with nucleic acids and their precursors indicate the importance of the purines. Nucleoside and nucleotide derivatives stain poorly, indicating that silver stains may require several functional groups to complex with a silver ion before its reduction to metallic silver.

Because of the presence of group-specific reactions, quantitative standards should be chosen with care, and quantitative inter-gel comparisons be limited to identical protein bands or spots. Similarity in running conditions (for example, percentage acrylamide and stacking gel specifications) may also be important as migration distance may affect band or spot compression, which in turn may influence the staining reactions.

Coloured silver stains

Silver staining can produce many colours. With appropriate conditions, lipoproteins may stain with a blueish hue, while some glycoproteins may stain yellow, brown or red⁸. This colour effect is due to the diffractive scattering of light by the microscopic silver grains⁹. While the development of polychromatic stains may complicate quantitative applications, silver staining protocols have been developed to enhance the development of colours, which may aid in the identification of proteins¹⁰.

Problems, artifacts, promises

Certain diamine stains may become selectively sensitive for glycoproteins if the concentration of silver ions is too low. This specificity can be minimized by maintaining a sufficient sodium-to-ammonium ion ratio in the diamine solution. However, some researchers have enhanced this tendency of the diamine stains to develop a protein stain that is selective for neurofilament polypeptides.

Artificial bands with molecular weights ranging from 50 to 68 kDa are often observed in silver-stained gels. Evidence has been presented indicating that these contaminating bands are due to keratin-type proteins¹¹. These bands may be enhanced by the presence of certain reducing agents, such as mercaptoethanol.

General background staining has been demonstrated to be due, in part, to the chemistry of the polyacrylamide gels.

Altering the chemistry of the gels by, for example, using special cross-linking agents, may result in reduced background¹⁴.

For unknown reasons, some proteins have proved to be difficult to stain with silver. Calmodulin and troponin C require pretreatment with glutaraldehyde, while histones require pretreatment with formaldehyde and Coomassie Blue. However, even with pretreatment, the sensitivity for histones is decreased as compared with detection of neural proteins.

While other metal-based stains that use copper, zinc and nickel, can be useful, they have not achieved the sensitivity of detection associated with silver stains. The colloidal gold stains provide a rapid method for protein staining membranes. They may also be linked to antibodies for the detection of specific proteins. However, their colloidal nature prohibits penetration of gel matrices, and this limits their applications to membranes.

Under optimal conditions, silver staining can detect 0.01 nanogram of protein or 200 million molecules of a 30-kDa protein. While our ability to detect proteins has increased over 100-million fold over the past 150 years, further increases in sensitivity may yet be possible. □

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Molecules on the move

Products for electrophoresis described this week include a 1- and 2-D gel analysis system, a temperature gradient gel electrophoresis system and an all-in-one crosslinker/transilluminator.

GEMINI is the new modular system from Joyce-Loebl for the **analysis of 1- and 2-D gels** (Reader Service No. 101). The \$39,000 (US) Gemini system is designed to digitize gels either from a high-speed drum scanner or from a range of high-resolution video cameras. Gemini consists of a central high-speed digital image processor with memory expansion potential to 4,096 × 4,096 pixels and a controlling processor that is PC-compatible. At the heart of the system is the Gemini software, which



Joyce-Loebl's Gemini system can separate spots from background and map differences in 1- and 2-D gels.

works in both interactive and automatic modes. It can be configured to process a 600-spot gel in 2–3 minutes, says Joyce-Loebl. The software can automatically process gels to separate the spots from background, removing streaks, smears and variations in density. Gemini can map the differences between up to 15 gels, and can output spot maps and spot occurrence diagrams to printers, ASCII files and commercial databases.

Dispense with the hazards and inconvenience of using radioactive ink to mark autoradiographs, Bel-Art Products is offering a handy pocket-sized **auto-radiography pen** (Reader Service No. 102). The pen uses a non-toxic, non-radioactive fluorescent material, which, Bel-Art says, will not leak, spill or dry out. The bright green markings do not glow in the dark, but will show up clearly on X-ray film.

To meet laboratory needs, Lab-Line Instruments has introduced the Model 3506 **reciprocal gel shaker** (Reader Service No. 103). The Model 3506 gel shaker features a variable stroke of 0.5–1.5 inch in 0.25-inch increments. Shaker speeds

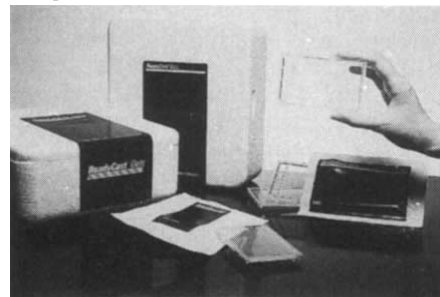


Model 3506: Lab-Line's versatile variable-speed shaker.

can be set between 10 to 250 motions per minute, running either continuously or in timed intervals of between one to 60 minutes. To ensure quiet operation, Labline says that all moving parts in the device are rubber mounted. Users can choose from a selection of interchangeable platforms in both dedicated and combination styles.

Pre-cast gels

Stop pouring gels by hand, says Ciba Corning, who now produces ReadyCast **analytical agarose mini-gels** for DNA electrophoresis (Reader Service No. 104). The 6.5 × 10 cm ReadyCast mini-gels are supplied with nine 15-μl pre-formed sample wells and are 4 mm thick. They are



Save time and effort and improve reproducibility of results with Ciba Corning's pre-cast DNase-free agarose gels.

suitable for the resolution of DNA fragments from 0.1 kb to 10 kb, and are compatible with most submarine minigel apparatus, including Hoefer, BioRad, Pharmacia and BRL gel boxes, says Ciba. ReadyCast gels contain 0.9 per cent DNase-free agarose and are available in either Tris-acetate-EDTA or Tris-borate-EDTA. For ease of handling and storage, ReadyCast gels are plastic-backed and have a shelf life of one year when unopened and stored at 18–25 °C.

For ease of preparation and reproducibility of results, Jule, Inc. recommends its new line of **polyacrylamide pre-cast gels** (*Reader Service No. 105*). Jule pre-cast gels are sold as 8 × 10 mini-gels or 16 × 18 large gels, and have a 4-month shelf life. They are compatible with most electrophoresis chambers and are available in either standard gradient or as gradient or non-gradient custom formulations. Each gel is individually cast, eliminating errors in batch processing, says Jule. To ensure protection during transport, each gel is shipped between two glass plates and individually packaged in a sealed foil pack. The mini-gels are sold in packs of eight with and without pre-formed wells for \$124 and \$112 (US), respectively, while the large gels are sold in packs of six without pre-formed wells for \$106 (US).

Power packs

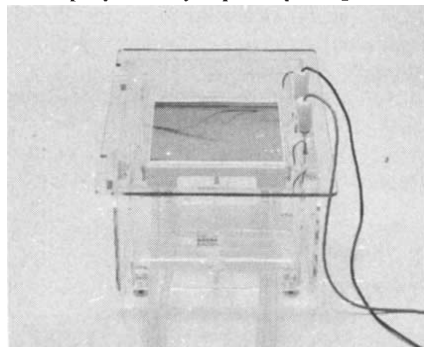
Jencons Scientific is launching a new line of **electrophoresis power supplies** to suit all laboratory needs (*Reader Service No. 106*). The Model E790 microcomputer pulsed-field power supply offers a programmable voltage ramp in both FIGE and OFAGE outputs, allowing the user to ramp both output voltage and pulse times (1 second up to 990 minutes). It provides, says Jencons, all the necessary control programs to separate large-sized DNA of more than 10 mb. With the E790, users can store up to six run programs. The E700 series comes in six models ranging in output from 200 to 5,000 V. These units provide constant voltage, current and power capabilities with automatic crossover. Up to 12 sets of parameters can be stored and manual, volt hours or time-controlled experiments can be run. The E400 microcomputer power supply also comes in six models with output ranging from 100 to 500 V, each of which has constant voltage and current capabilities with automatic crossover.

Fisher Scientific has launched a full line of **power supplies** to meet all horizontal and vertical electrophoretic applications (*Reader Service No. 107*). All models feature solid-state circuitry, which helps eliminate drift caused by temperature changes and ageing components, and allows the units to operate at 0–30 °C, says Fisher. The 'constant power' units operate in any of three modes: constant power, current or voltage. Designed primarily for IEF and DNA sequencing, the units range in output from 1,000 to 6,000 V. The 'constant current' units operate in two modes: constant current and voltage. All three power supplies have an output of 500 V and are recommended for vertical electrophoresis techniques. The remaining five models are 'constant voltage' units. They have outputs ranging from 25 to 500 V and are recommended for submarine gel electro-

phoresis and gel destaining. As a safety feature, all models with output voltages greater than 250 V, have a shutdown-on-cell-disconnect circuit, which turns off the voltage if the leads are disconnected from the gel chamber.

Electrophoretic systems

According to Diagen, mutations and polymorphisms can be directly analysed on its new **temperature gradient gel electrophoresis system** (TGGE) (*Reader Service No. 108*). TGGE is designed to separate biopolymers by superimposing a linear



Diagen's new TGGE system for analysing mutations and polymorphisms.

temperature gradient perpendicular or in parallel to the electric field. The denaturing temperature gradient is, says Diagen, easier to handle and produces more reproducible results than denaturing chemical gradients. TGGE can be used to monitor the genetic stability of expression systems in research and development and quality control, detect polymorphisms of homologous gene loci without the need for blotting and hybridization procedures, and detect single-point mutations on small (100 bp) and large (1 kbp) DNA fragments.

Femtomole detection sensitivity, multiple sample injection modes and a choice of automated or manual operation are features of the two new **capillary electrophoresis systems** from Isco (*Reader Service No. 109*). Both the automated

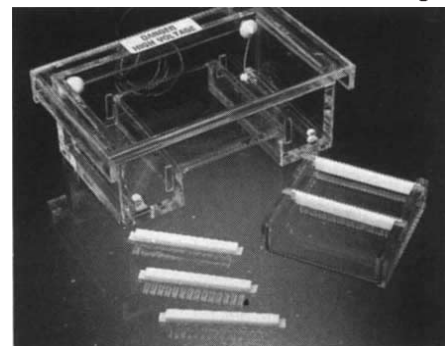


Isco offers complete capillary electrophoresis systems.

multi-sample instrument and the compact single-sample instrument can be used for free-zone HPCE of peptides, proteins and polynucleotides, as well as surface-modified capillaries, and for micellar electrokinetic capillary chromatography and electrofocusing techniques, says Isco. The automated system includes a 40-position autosampler, computer-control over

separation parameters such as buffer switching and voltage, and data management facilities. The micro-collection of separated fractions will be available in the future. The manual system includes a syringe-loading injector that requires less than 3 µl of sample per analysis and a built-in 30-kV power supply with switchable polarity.

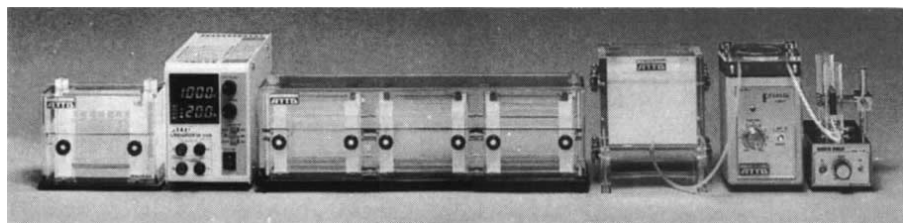
The Schleicher and Schuell Sub 0911 **horizontal electrophoresis chamber** is supplied with everything you need to cast, run and view gels (*Reader Service No. 110*). Made of a heavy-duty acrylic material, the Sub 0911 chamber accommodates a 9 × 11 cm gel bed, and is suitable for restriction fragment analysis, says S & S. Gels are cast *in situ*: the removable UV-transparent gel tray is turned sideways and sealed against the inner walls of the chamber. To run a gel, the tray is turned 90°. Corrosion-resistant platinum electrodes, power cables, a vented cover for the insertion of buffer recirculation tubing, levelling screws, a bubble level, and 10- and 14-tooth 1.5-mm thick gel



S & S's mini horizontal electrophoresis system — all you need to cast, run and view gels.

combs are provided as standard. As a safety feature, the slide-on safety lid must be in place for the unit to operate. An 18-tooth 1.5-mm gel comb, 1.5-mm wall comb and 1.5-mm preparative comb are optional extras.

The Investigator **2-D electrophoresis system** is Millipore's protein separations system which uses large-format gels (*Reader Service No. 111*). Emphasizing the advantages of using the larger gel format, Millipore says proteins are more efficiently dispersed and more information is provided for analysis than with standard 2-D gels. The Investigator 2-D system is supplied with a programmable power supply for storing sets of running conditions; a patented threaded tube 1-D casting system for providing consistent gel lengths, without inconsistencies such as stretching; and a chiller for maintaining the temperature of slab gels at a constant 20 °C during second-dimensional electrophoresis. Millipore says, the temperature control feature helps produce clearly defined spots, as migration and gel distortion are kept to a minimum.



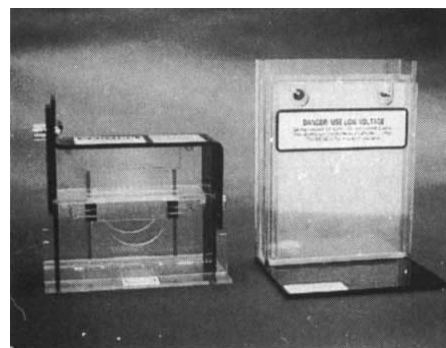
Rectangel — GRI's mini vertical PAGE system runs six rod gels simultaneously.

Rectangel is the **mini vertical PAGE system** from Genetic Research Instrumentation designed for the rapid separation of protein in multi-samples (*Reader Service No. 112*). Rectangel is a three-part system. First, there is a tube gel chamber for first-dimensional IEF. Using the apparatus, six rectangle-shaped tube gels are prepared in a slab arrangement. By positioning a special glass plate with spacers bonded to it over the top plate, six rod gels are formed measuring 1 mm × 2 mm × 80 mm. Second, is a chamber that accepts up to six mini gels. Glass plates are positioned using pressure plattens — no slips, screws or tape are needed, says GRI. The gel plates are positioned directly against the anode buffer, which eliminates 'smile' effects and allows gels to run fast with minimum heat build-up. A bevel on the glass plate allows the rectangle tube gel to be positioned accurately for the second-dimensional electrophoresis. Third, is a mini gel casting tower for preparing up to eight mini gels — gradient or standard — simultaneously.

Gradipore of Australia is offering a new system called Micrograd for **gradient, SDS and 2-D electrophoresis, and IEF** (*Reader Service No. 113*). Micrograd, costing \$625 (Australia), is available in two models: the MG-001 standard system and the MG-002 with attached cooling system for extended runs. Gradipore emphasizes that the main advantage of Micrograd is speed: runs that use to take 2-3 hours now take less than 15 minutes. The reasons for this are two-fold: the distances molecules travel before reaching the 'pore limit' is about three times shorter than with standard gels, and the short gel length leads to an increase in

the electric field strength. Micrograd consists of two moulded acrylic tanks. The inner tank housing the platinum electrodes has a patented sealing system and holds 250 ml of buffer. The outer tank holds 750 ml of buffer and can be fitted with an external cooling system to improve heat dissipation. The tanks are locked together by the lid. The key to the Micrograd system is the range of pre-cast gels that Gradipore offers, which can be run in pairs with the Micrograd system.

ARH Horwell is distributing Idea Scientific's Minislab **mini electrophoresis system** (*Reader Service No. 114*). Minislab



Minislab vertical gel chamber and Genie electroblotter — new from ARH Horwell.

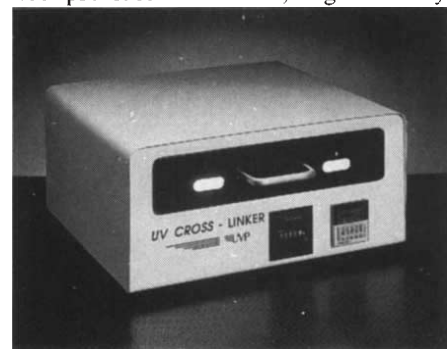
uses 8 × 10 cm or 10 × 10 cm glass plates for vertical gel electrophoresis with 18-well combs. Typically, electrophoresis takes 50-90 minutes, with short staining and destaining times because the gels are thin, says the company. Single bands can be detected at the 5-10-nanogram sensitivity level with Coomassie blue and 100-picogram level with silver staining. Bands can then be blotted onto paper using the Genie Electroblotter. Genie requires low voltage and provides a uniform electric field, producing, says the company, undistorted blots without the problem of air bubbles.

Jordan Scientific has introduced the PhorRunner **dual mini-gel system**, which is designed for the rapid screening of protein and nucleic acid samples (*Reader Service No. 115*). PhorRunner can run one or two 7 × 8 cm polyacrylamide gels in 45 minutes and can be used with pre-cast gels, says Jordan. The system is equipped with thick gel plates and machined PVC screw clamps to help prevent plate breakage during set-up and use. PhorRunner also features an anti-condensation safety cover and gel-casting stand for gel pouring.

DNA/RNA crosslinkers

Bios Corporation announces the Bioslink series of **linker and linker/transilluminator all-in-one units** for DNA or RNA fixation to nylon membranes with UV light (*Reader Service No. 116*). The Bioslink units are available with either 254-nm or 312-nm bulbs, and with or without a transilluminator for DNA or RNA visualization. However, 312 or 312T units can be converted to the 254-nm wavelength by simply changing the bulb. The 254 or 254T units can be used for crosslinking nucleic acids or in other situations where UV treatment is required, while the 312 and 312T units provide fast crosslinking, minimizing DNA cleavage caused by overexposure to UV, says Bios. All units are equipped with a detection cell that monitors the amount of energy applied to the membrane. Energy levels can be pre-set. When the pre-set level is reached, the energy-monitoring cell automatically shuts off. As optimal energy is applied to blots, binding is completed in seconds, producing strong hybridization signals, says Bios, which is important for blots that are probed many times.

Ultra-Violet Products has a new **cross-linker** — the CL-400 — which can be used to transfer DNA and RNA fragments to Hybond-N membranes from electrophoresis gels (*Reader Service No. 117*). The gel is placed in the drawer, the UV exposure is set on the timer and the system automatically shuts down when the exposure level has been reached. The CL-400 provides a uniform, high-intensity



HV crosslinker: UVP's DNA/RNA transfer tool.

shortwave UV light (254 nm) to transfer DNA (Southern) and RNA (Northern) fragments to Hybond membranes. The user can choose from eight time modes, and an LCD displays the time while the crosslinker is in operation. For user protection, a safety interlock prevents operation unless the gel drawer is fully closed and special filters ensure the user is not exposed to harmful UV. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. To obtain additional information about these products, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.

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